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# **CANINE MODELS OF HUMAN VISION DISORDERS: IDENTIFICATION OF NEW LOCI AND GENES FOR GLAUCOMA AND RETINAL DEGENERATION**

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*To my beloved grandmother*

# ABSTRACT

The recent development of canine genomic resources combined with the unique phenotypic diversity, population history and breed structure has made dogs as exciting large animal models for genetic research. Dogs suffer from hundreds of hereditary conditions, many of which are similar to human disorders.

Dog breeds are affected with various hereditary, blinding eye diseases, such as cataract, glaucoma, retinal degeneration and lens luxation. Although a limited number of genes have been found in some conditions, many more remain to be found across breeds. Gene discoveries are important in improving the understanding of related disease mechanisms and eye physiology, informing breeding programs, and establishing new therapeutic models. This study utilized and expanded the remarkable canine resources established in Finland to reach new genetic breakthroughs in four different canine hereditary vision disorders including retinal degeneration and glaucoma in different breeds.

This study combined basic and clinical research with a network of collaborations with breeders and veterinarians to establish clinically relevant study cohorts for genetic studies. Genome wide approaches with modern sequencing technologies were utilized to map and identify the genetic causes. Study cohorts were established for a late-onset progressive retinal atrophy (PRA) in the Papillons and Phalènes, for retinopathy in the Swedish Vallhunds (SV) and primary closed and open angle glaucoma in Dandie Dinmont Terrier (DDT) and Norwegian Elkhounds (NE), respectively.

Genome wide association study (GWAS) mapped the PRA gene in Papillons and Phalènes to canine chromosome 2 (CFA2) and parallel exome sequencing identified a breed-specific, recessive mutation (p.Tyr889Serfs\*5) in the *CNGB1* gene with a high carrier frequency (17 %). *CNGB1* is important for the rod cell function and has been linked to human and murine retinal degenerations.

Clinical studies in the Swedish Vallhund (SV) breed revealed a unique type of rod- or RPE-derived multifocal retinal degeneration, with variable onset and rate of progression and an incidence of 35 %. The disease was mapped to a 6-Mb region on CFA17, with the most highly associated variant found in the intron of the *MERTK* gene with a carrier frequency of almost 60 %. Quantitative analysis revealed specific upregulation of the gene, indicating that the retinopathy is caused by overexpression of *MERTK*. However, further studies are needed to discover the actual mutation. *MERTK* is an excellent candidate gene as it has been associated with similar retinal degeneration in rats and humans.

Glaucoma is related to neurodegeneration mainly of the retinal ganglion cells (RGC) and of the optic nerve head. Elevated intra ocular pressure (IOP) is a significant risk factor and in dogs, pectinate ligament dysplasia (PLD) is commonly associated with glaucoma.



A clinical study in DDTs revealed primary closed angle glaucoma (PACG) with severe PLD. The GWAS revealed a significant association with a 10-Mb region on CFA8, which is syntenic to human chromosome 14 that has been associated repeatedly with glaucoma. The identified region includes a large number of genes and further studies are required to identify the causative mutation in DDTs.

Finally, primary glaucoma in NEs was mapped to a region on CFA20 and a missense mutation, p.A387T, was found in a highly conserved metalloprotease domain of the *ADAMTS10* gene. This gene has been associated with a Weill-Marchesani syndrome (WMS) with ocular and non-ocular abnormalities in humans and primary open angle glaucoma in the Beagle breed.

Overall, this study brought several breakthroughs and produced new insights into the genetics of two blinding ocular diseases in dogs. Gene discoveries highlight shared molecular etiologies in canine and human vision disorders. This study provides new candidate genes for human conditions and has established large animal models for potential therapeutic trials, including gene therapy. Genetic findings have enabled the development of gene tests for breeding purposes. Furthermore, this study contributes to the development of additional clinical, methodological, and sample recruitment resources for further genetic studies in many other canine eye disorders beyond the addressed conditions.

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# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications.

- I            **Ahonen SJ**, Arumilli M, Lohi H (2013). A *CNGB1* Frameshift Mutation in Papillon and Phalène Dogs with Progressive Retinal Atrophy. PLoS ONE 8(8): e72122.
- II           **Ahonen SJ**, Pietilä E, Mellersh CS, Tiira K, Hansen L, Johnson G, Lohi H (2013). Genome-Wide Association Study Identifies a Novel Canine Glaucoma Locus. PLoS ONE 8(8): e70903.
- III          Cooper AE, **Ahonen SJ**, Rowlan JS, Duncan A, Seppälä EH, Vanhapelto P, Lohi H, Komáromy AM (2014). A Novel Form of Progressive Retinal Atrophy in Swedish Vallhund Dogs. PLoS ONE 9(9): e106610.
- IV          **Ahonen SJ**, Arumilli M, Seppälä EH, Hakosalo O, Kaukonen MK, Komáromy AM, Lohi H (2014). A Unique Form of Canine Retinopathy is Caused by Upregulation of *MERTK*. PLoS ONE, in press.
- V           **Ahonen SJ**, Kaukonen M, Nussdorfer FD, Harman CD, Komáromy AM, Lohi H (2014). A Novel Missense Mutation in *ADAMTS10* in Norwegian Elkhound Primary Glaucoma. PLoS ONE, in revision.

The publications are referred to in the text by their roman numerals.

# ABBREVIATIONS

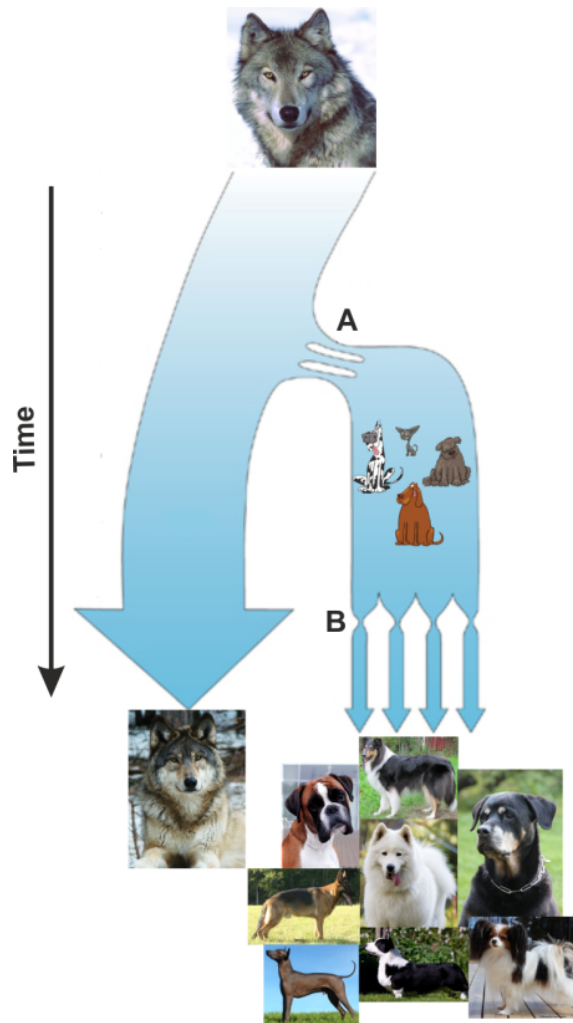
BVMD	best vitelliform macular dystrophy	OMIA	Online Mendelian Inheritance in Animals
CEA	Collie eye anomaly	OMIM	Online Mendelian Inheritance in Man
CFA	canine chromosome	ONH	optic nerve head
cGMP	cyclic guanosine monophosphate	ONL	outer nuclear layer
chr	human chromosome	OPL	outer plexiform layer
CNG	cyclic nucleotide gated	OS	outer segment
CRD	cone-rod degeneration	PAP	Papillon
CSNB	congenital stationary night blindness	PCAG	primary closed angle glaucoma
DDT	Dandie Dinmont Terrier	PCG	primary congenital glaucoma
ECM	extracellular matrix	PCR	polymerase chain reaction
ECVO	European College of Veterinary Ophthalmologist	PHA	Phalène
ERG	electroretinogram	PL	pectinate ligament
FCI	Fédération Cynologique Internationale	PLD	pectinate ligament dysplasia
GCL	ganglion cell layer	POAG	primary open angle glaucoma
GWAS	genome wide association study	PR	photoreceptor
HC	hereditary cataract	PRA	progressive retinal atrophy
HRUS	high-resolution ultrasonography	qRT-PCR	quantitative real time PCR
ICA	iridocorneal angle	ROS	rod outer segment
INL	inner nuclear layer	RWOCC	relative width of the opening of the ciliary cleft
IOP	intra ocular pressure	RGC	retinal ganglion cell
IPL	inner plexiform layer	RP	retinitis pigmentosa
IS	inner segment	RPE	retinal pigment epithelium
LCA	Leber Congenital Amaurosis	rAAV	recombinant adeno-associated virus
LD	linkage disequilibrium	SINE	short interspersed nuclear element
MAF	minor allele frequency	SNP	single nucleotide polymorphism
mtDNA	mitochondrial DNA	SNV	single nucleotide variant
NB	night blindness	SV	Swedish Vallhund
NE	Norwegian Elkhound	TGFβ	transforming growth factor beta
NFL	nerve fibre layer		
NGS	next generation sequencing		
NMD	nonsense mediated decay		
OCT	optical coherence tomography		

All gene names and symbols can be found in the HGNC database ([www.genenames.org](http://www.genenames.org)).

# 1 REVIEW OF THE LITERATURE

Purebred dogs have a unique population history. Dogs (*Canis lupus familiaris*) descend from the grey wolf (*Canis lupus lupus*) and have undergone multiple bottlenecks during the domestication process (Fig. 1). The timing and location of dog domestication remains controversial. The first mitochondrial DNA (mtDNA) based studies suggested the origin to go back 100,000 years<sup>1</sup>, while this estimation was cut to 16,000 years in later studies<sup>2,3</sup>. The fossil evidence of early dog-like canids suggests the domestication event to have taken place between 11,000 to 30,000 years ago<sup>4,5</sup>. Thalmann et al. sequenced archeological samples and concluded an European origin of dogs. However, their study lacked a reasonable representation for the East Asian population<sup>6</sup>. A recent nuclear DNA sequencing study suggested that the domestication took place about 11,000 to 16,000 years ago with the hunter-gathers rather than agriculturists and that the dog-wolf admixture has had an effect in the domestication process<sup>7</sup>. Furthermore, it has been suggested that the canine evolution has been parallel with the human evolution<sup>8</sup>. In addition, there were probably multiple domestication events in multiple places that led to various domesticated lineages<sup>7</sup>. Further studies with more comprehensive worldwide cohorts including additional archeological samples are needed for more accurate conclusions.

Most of the dog breeds today have been created during the last 100-400 years with strong founder and breed barrier rule effects, including frequent use of popular sires. Breed barrier rules define that only dogs that are born from parents of a single specific breed can be registered as purebred. Today purebred dogs are divided into more than 400 different breeds, representing high phenotypic variability between breeds in appearance, behavior and genetic diseases. Breeders have taken advantage of spontaneous mutations rather than depending on traditional methods of mutagenesis, such as chemicals or retroviral insertions<sup>9</sup>.



**Figure 1.** The dog has been created from the grey wolf through multiple bottlenecks, from wolf to ancient breeds (A) and to modern breeds with variable appearances (B). This unique population structure facilitates genetic studies.

## 1.1 Unique breed and genome structure facilitates gene mapping in dogs

Every dog breed forms a distinct, genetically isolated population, with limited gene flow due to breed barrier rules. In addition, modern breeding practices often focusing on a particular appearances present in champions modify the genetic structure and diversity of the current breeds.

The unique population structure makes the dog an excellent model animal for genetic research. Limited locus and disease heterogeneity accompanies the creation of hundreds of breeds with distinct morphological and behavioral features<sup>10-12</sup>. Within a breed, the genetic diversity is limited and characterized by an extensive linkage disequilibrium (LD), narrow diversity in haplotypes and broad runs of homozygosity<sup>10,11</sup>. Each breed has a unique recombination history and a breed-wise comparison of the genomes reveals varying ancestral

haplotypes that can be utilized in gene mapping to narrow down the region of interest<sup>10,13</sup>.

Along with the creation of dog breeds, the prevalence of hereditary disease has increased in different breeds. Over 600 genetic disorders have been described in dogs<sup>14</sup>. Most conditions are breed-specific and the phenotypic spectrum is often more limited than in the corresponding human conditions. Of the 600 diseases listed in the Online Mendelian Inheritance in Animal database (OMIA)<sup>14</sup>, a causative mutation has been found for over 180 conditions. Many conditions diagnosed in dogs are analogous to human diseases, such as cataract, diabetes, epilepsy, glaucoma, and retinal diseases<sup>14</sup>. The dog and the human share a 95% genetic similarity, which is higher than between humans and rodents. Several findings in dogs have paved the way to unravel the genetic in humans conditions such as cancer<sup>15,16</sup>, eye disease<sup>17</sup>, and neurological diseases<sup>18</sup>. Besides similar disorders, pet dogs share their environment with humans, becoming exposed to the same environmental factors as humans. This is a remarkable contrast to common rodent and fish models, which live in highly controlled and pathogen-free laboratory environments.

### **1.1.1 Tools and resources for gene mapping in dogs**

The availability of genealogical data facilitates canine genetic studies. As breed purity necessitates the generation of breed-specific records, dogs can often be traced back to several generations. In Finland, the Finnish Kennel Club maintains a publicly available database ([jalostus.kennelliitto.fi/](http://jalostus.kennelliitto.fi/)), which contains pedigree and health information, including eye examination data, of individual dogs. These records help to establish large pedigrees around the affected dogs to model inheritance patterns and to design genetic studies.

The first whole canine genome sequence was published in 2003 based on a 1.5x sequence from a Standard poodle<sup>19</sup>, followed by a higher quality 7.5x version from a Boxer genome (CanFam1.0). This was later updated to CanFam2.0 assembly in 2005<sup>10</sup>. This version, although of good quality, included gaps, particularly in the promoter region impacting the annotation of the genes. The most recent version (CanFam3.1) based on the whole genome sequence from multiple breeds was published in 2012. It covers 99.8 % of the euchromatin and includes RNA sequences from 10 different canine tissues<sup>20</sup>. The CanFam3.1 genome is an improved version, which includes a comprehensive annotation of protein coding and non-coding genes, including different isoforms<sup>20</sup>. The current genome of 2.4 Gb is divided into 39 chromosomes and includes approximately 19,900 protein coding genes and 29,900 transcripts<sup>20</sup>.

Based on the canine genome reference sequence three single nucleotide polymorphism (SNP) arrays have been developed for genome



wide case-control association studies (GWAS). SNP is a DNA sequence variation that is commonly seen in a population, in which a single nucleotide differs between individuals. GWAS analysis is performed by comparing allele frequencies of each SNP. The aim is to find statistically significant differences between the case and control group of choice. The first SNP array was developed by Affymetrix and included 50,000 SNPs, followed by two Illumina arrays, Illumina Canine SNP20 BeadChip, including 22,000 markers, and the current state-of-the-art Illumina CanineHD BeadChip with 173,000 SNPs. Extensive LD within breeds makes it possible to test the genome with fewer SNPs than required in human studies<sup>10</sup>. In dogs multiple loci of large effect have been described for several phenotypes using GWAS<sup>9</sup>. However, extensive LD within breeds makes fine mapping more difficult, as many markers co-segregate with the actual causal variant<sup>9</sup>.

Besides SNP arrays, the advanced next generation sequencing (NGS) approaches can be applied to canines. Both the targeted and whole exome sequencing approaches are based on probes designed for the canine reference sequence. In exome sequencing specific probes are designed to target the protein coding genes, which are selectively captured from the whole genome and sequenced. Two specific platforms are available for canine whole exome sequencing. The first exome capture kit was developed by Agilent (Agilent, Santa Clara, CA, USA) based on the CanFam2.0 reference sequence. The capture includes a 54 Mb design that covers the Ensembl ([www.ensembl.org](http://www.ensembl.org)) and RefSeq Genes from the UCSC track ([genome.ucsc.edu](http://genome.ucsc.edu)), human protein alignments and spliced ESTs that lie outside Ensembl. Another exome capture kit, SeqCap EZ Library, is offered by Roche Nimblegen and is based on a solution-based capture method and a custom design of approximately 50 Mb, based on the CanFam3.1 reference sequence designed in the Broad Institute ([www.broadinstitute.org](http://www.broadinstitute.org)).

The disadvantage of NGS relates to more complicated approaches in data quality, analysis and management. Millions of SNPs and indels are produced from the variant calling and the number of possible candidate variants may be very high. Especially in more complex phenotypes where the causative variant may lie outside the protein coding regions. However, the more dogs are whole genome and exome sequenced and the data, with good coverage, made publicly available, the more efficient filtering may be applied to the data to decrease the number of potential causative variants.

These modern tools have been utilized in this study to unravel genetic causes in canine vision disorders.

## **1.2 The eye**

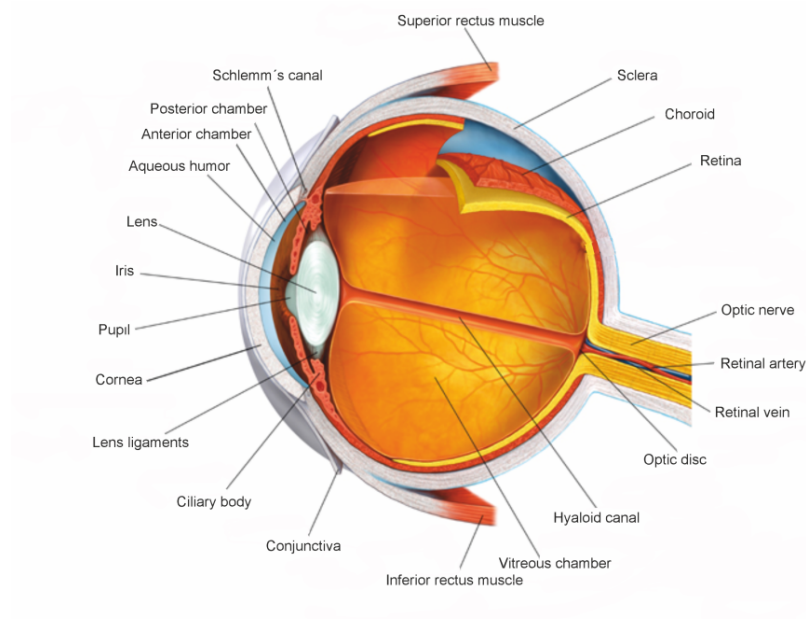
The eye is a very unique sensory organ responsible for vision (Fig. 2). It is designed to function in a camera like fashion and to absorb and reflect light<sup>21</sup>. The light is directed to the retina and electro-chemical impulses are sent through complex neural pathways via the optic nerve to the visual cortex, where nerve signal is conferred to a visual image<sup>21</sup>.

Vertebrates have complex eyes of different shapes and colors. Animals with binocular vision, especially predators, have eyes that move simultaneously to improve depth perception<sup>22</sup>. Animals with monocular vision have eyes that are used separately to see and are positioned widely apart as possible<sup>22</sup>. In addition different species show huge variation in such aspects of vision as visual acuity, color vision, pigmentation, light sensitivity, and the numbers and types of photoreceptor cells in the retina<sup>22</sup>.

The normal structures of the canine eye and the diseases affecting the various parts of the eye are probably the best described and characterized in dogs among model animals used in genetic research<sup>17</sup>. The eye is a very accessible organ and non-invasive techniques can be used to diagnose and treat abnormalities affecting different parts of the eye<sup>17</sup>. However, the eye is also a very sensitive organ and diseases in other parts of the body may cause abnormalities in the eye. For example, diabetes may cause diabetic retinopathy, secondary cataract, and glaucoma<sup>23</sup>. Similarly, inflammation may cause retinal and lens alterations and glaucoma<sup>24,25</sup>.

Hereditary eye diseases affect primarily a certain part of the eye, where diseases of the retina and lens are the most prevalent<sup>17</sup>. For example, retinal degenerations originate from the abnormal function and structure of particular retinal cells<sup>26</sup>. In cataracts, abnormalities are detected in the lens structure<sup>27</sup> and primary lens luxation is due to the deterioration of the lens ligaments that hold the lens in its position rather than actual disease of the lens<sup>28</sup>. Furthermore, glaucoma is characterized by neurodegeneration of the RGCs and changes in the optic nerve head<sup>29</sup>. Moreover, many other diseases affecting other parts of the eye are diagnosed. However, many eye diseases lead to secondary manifestations in other parts of the eye<sup>30</sup>.

As this study focused on the genetics of retinal degeneration and glaucoma the specific characteristics of the eye that are affected will be discussed in more detail in the following chapters. These characteristics include the structure of the retina, the visual cycle, which is related to the function of the retina, and the iridocorneal angle and aqueous humor flow that are related to the development of glaucoma.

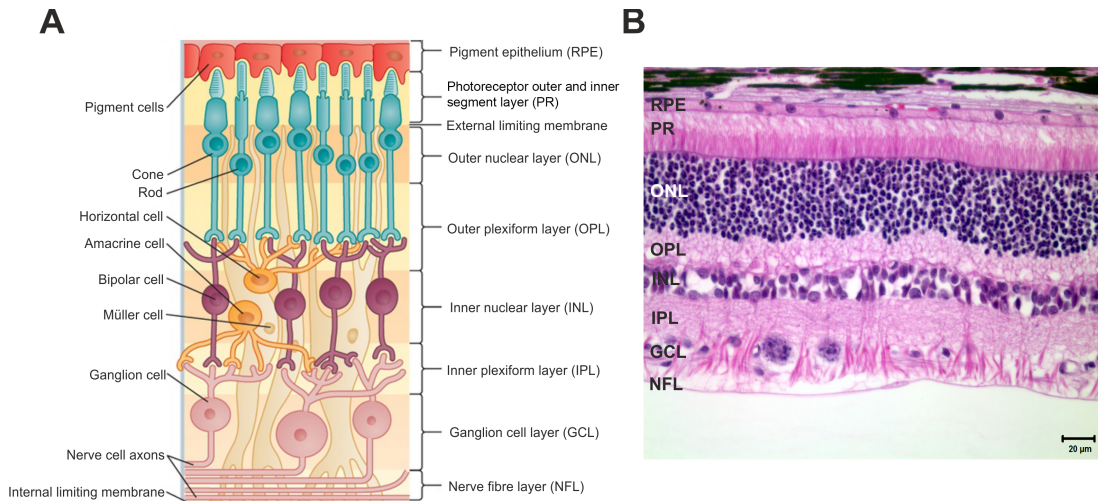


**Figure 2.** A schematic structure of the eye and its functional components (modified from virtualmedicalcenter.com).

### 1.3 Retina

The visual process is generated through multiple biochemical and photochemical reactions<sup>21</sup>. These light sensitive reactions take place in the retina, which is a highly organized structure and part of the central nervous system (Fig. 3)<sup>21</sup>. The retina is a complex tissue in the back of the eye. Newborn canines have an undeveloped retina that matures during a postnatal period at 3-6 weeks<sup>31</sup>. The structure of the canine and human retina is similar, except that humans are born with a more developed retina. In addition, dogs have a light-reflecting area called tapetum lucidum and a heavily pigmented choroid but lack the macula and fovea<sup>32</sup>. However, dogs have a fovea-like area called *area centralis*, which is constructed with uniquely packed cone photoreceptors<sup>33</sup>.

The retina can be divided into tapetal fundus backed by brightly reflecting tapetum lucidum and non-tapetal fundus<sup>32</sup>. The optic disc is in a fixed position in the area where the optic nerve exists the orbit<sup>22</sup>. The color of the retina varies greatly between breeds and in some breeds is related to the coat color (Fig. 4)<sup>34</sup>. The color of the retina does not appear to have an effect on the eye function<sup>34,35</sup>.

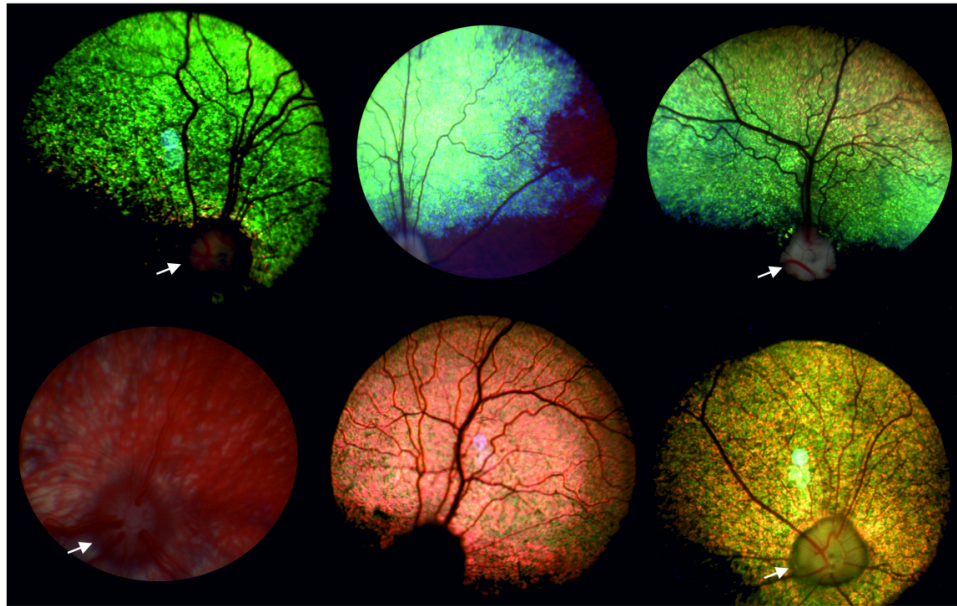


**Figure 3. A)** A schematic description of the retinal layers (modified from Koeppen & Stanton: Berne and Levy Physiology, 6<sup>th</sup> Edition). **B)** Histology of the normal canine retina shows clearly the different layers of the retina (photo courtesy of Professor Andras Komáromy). In retinal degeneration the photoreceptors, rod and cones, are typically affected. Glaucoma is caused by degeneration of the retinal ganglion cells (RGC).

The neuroretina is supported by the retinal pigment epithelium (RPE) and the Müller glial cells. The latter cell types also provide important metabolites to the photoreceptors and act as scavengers by removing used photopigments. There are six different types of neuronal cells in the retina: ganglion, amacrine, bipolar, horizontal, interplexiform, and photoreceptor cells. All are involved in light-processing (Fig. 3)<sup>21</sup>. Traditionally, the retina is divided into 10 different layers: retinal pigment epithelium (RPE), photoreceptor layer, external limiting membrane, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, nerve fibre layer and internal limiting membrane (Fig. 3)<sup>21</sup>.

Photoreceptor cells comprise a complex layer of specialized cells, rods and cones. Rods function in low light and are inactivated by constant bright light<sup>21,22</sup>. Cone cells function during daylight, adapt rapidly and need strong light stimulus to react<sup>21,22</sup>. They do not function in dim light<sup>21,22</sup>. The photoreceptor cell can be subdivided into the following parts; the inner and outer segments, nucleus and the synaptic structure. The inner and outer segments are connected by a connecting cilium originating from the basal body in the inner segment<sup>21</sup>.

The optic nerve head appears as cream or pink colored mainly circular area in the fundus (Fig. 4)<sup>21</sup>. It is responsible for the transfer of light induced signals from the retina to the visual cortex in the brain<sup>36</sup>. The optic nerve is mainly composed of axons of the RGC<sup>36</sup> and includes four regions, the RGCs, the nerve fibre layer (NFL), the optic nerve head (ONH) and the intralaminar region within the sclera (Figs. 2 and 4)<sup>21</sup>.

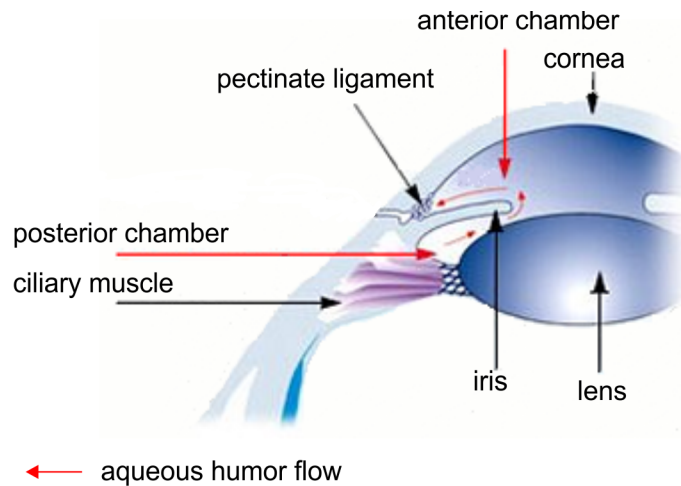


**Figure 4.** A large variation is detected in the color of normal canine retina between different breeds (photos DVM Sari Jalomäki and DVM Kaisa Wickström). The optic nerve head is marked with an arrow.

## 1.4 The iridocorneal angle and aqueous humor flow

The iridocorneal angle (ICA) is formed circumferentially by the peripheral parts of the iris and cornea (Figs. 2 and 5)<sup>21</sup>. The internal boundary of the ICA is formed by pectinate ligaments (PL) structure that is not present in the human eye (Fig. 5)<sup>37</sup>. The PL is presented as a pillar of tissue, which projects from the base of the iris to the peripheral Descemet's membrane and provides support for the iris toward the posterior cornea<sup>37</sup>. The normal pectinate strands are thin to stout strands bridging the iridocorneal angle from the iris base to the periphery of the cornea<sup>38</sup>. Most of the strands are single, although some may be connected<sup>38</sup>. Normal PLs do not form an anatomic barrier to aqueous humor flow<sup>37</sup>. Behind the PL is a matrix of loose tissue strands, the trabecular meshwork, which consists of crisscrossing collagen cords<sup>21</sup>. The space between PLs is filled with glycosaminoglycans (GAG)<sup>37</sup>, which form the integral component of trabeculae within the ICA. GAGs appear to regulate IOP by their state of polymerization. Abnormalities in the PLs may cause the release or abnormal polymerization of GAGs<sup>21</sup>.

Aqueous humor flows from the posterior chamber through the pupil into the anterior chamber, and to the drainage angle (Fig. 5)<sup>21</sup>. It flows between the PLs and into the trabecular meshwork. The aqueous humor leaves the eye either through the corneoscleral trabecular meshwork or the ciliary body and anterior uvea<sup>21</sup>. When the aqueous humor flow production by the ciliary body and drainage through the ICA is in balance it creates a normal IOP, helping to maintain the shape of the eye<sup>21</sup>.



**Figure 5.** The schematic structure of the iridocorneal angle (ICA). The aqueous humor flows from the posterior into the anterior chamber, and to the drainage angle. It flows between the pectinate ligaments and into the trabecular meshwork (picture modified from Holger Funk: [www.shiba-dog.de/shiba-klub/glaukom-en.htm](http://www.shiba-dog.de/shiba-klub/glaukom-en.htm)). In closed angle glaucoma abnormalities in the pectinate ligaments lead to obstruction of the aqueous humor and collapse of the ICA, which further leads to elevated IOP.

## 1.5 Visual cycle

The visual cycle occurs mainly in the RPE, Müller cells, and in the photoreceptors<sup>39</sup>. Two important pathways, the retinoid cycle and the phototransduction cascade, are involved in vision (Fig. 6)<sup>39</sup>. The rod dominated retinoid cycle starts with the absorption of light by rhodopsin<sup>39</sup>. This causes 11-*cis*-retinal to bind as protonated Schiff base to rhodopsin and isomerize to all-*trans*-retinal, producing inactive protein opsin<sup>39</sup>. It is then detached from rhodopsin in photoreceptor cells and enzymatically transformed by retinol dehydrogenase (RDH) to all-*trans*-retinol (vitamin A) in the rod cells<sup>40</sup>. Vitamin A is then transported from photoreceptors into the RPE by retinol binding protein (IRBP)<sup>40,41</sup> or by ABCA4 transporter<sup>42</sup>. After this, it is transferred to lecithin retinol acyl transferase (LRAT)<sup>43,44</sup> by cellular retinol-binding protein (CRBP). LRAT is esterified and further hydrolyzed and isomerized by retinal pigment epithelium 65 (RPE65) and other enzymes and retinoid binding proteins to produce 11-*cis*-retinol<sup>39,45</sup>. The light sensitive 11-*cis*-retinol is oxidized to 11-*cis*-retinal by *cis*-specific retinol dehydrogenase (*cis*-RDH5). This cascade is chaperoned by cellular retinaldehyde binding protein (CRALBP)<sup>39,45</sup>. The 11-*cis*-retinal is then taken up by CRALBP and transported to the apical membrane of the RPE and transferred back to the photoreceptor outer segment. It is then reattached to opsin to form functional rhodopsin (Fig. 5)<sup>39,45</sup>.

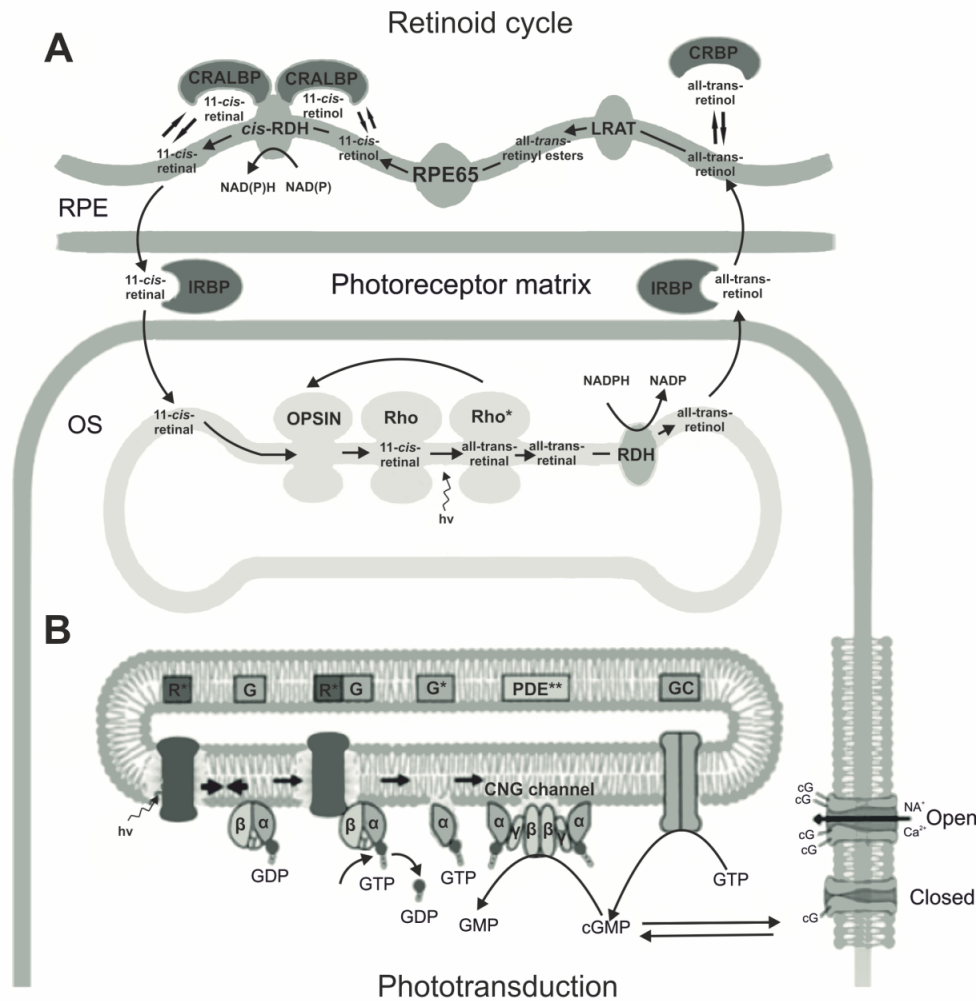
However, the retinoid cascade is more complex and alternate pathways have been reported<sup>46,47</sup>. In addition, additional enzymes have been

identified that play a role in the visual cycle<sup>48</sup> and in the mouse the presence of other *cis*-retinals has been shown<sup>48,49</sup>.

The phototransduction cascade functions in the conversion of light stimuli to neuronal signal (Fig. 6). The cascade is activated by the absorption of light by the visual pigment which activates G-protein signaling cascade<sup>50,51</sup>. The heterotrimeric G-protein transducing (Gt) is bound to rhodopsin (R\*) and this in turn activates phosphodiesterase (PDE) to PDE\*\*<sup>52</sup>. PDE\*\* hydrolyzes intracellular cGMP<sup>52</sup>. This leads to the closure of the cGMP-gated ion channels in the plasma membrane and the subsequent reduction in intracellular calcium ( $\text{Ca}^{2+}$ )<sup>53</sup>. This leads to hyperpolarization of the photoreceptors and further to neuronal signalling<sup>53</sup>. The decrease in  $\text{Ca}^{2+}$  concentration induces a conformation change in recoverin (Rec) and its dissociation from rhodopsin kinase (RK)<sup>54</sup>. After that the kinase can bind and multiply phosphorylated rhodopsin<sup>54</sup>. The increased rhodopsin level decreases the binding affinities of RK and Gt and increases the affinity of Arrestin (Arr) for rhodopsin<sup>54</sup>. The decrease in  $\text{Ca}^{2+}$  also triggers the activity of two guanylate cyclase activating proteins (GCAPs). This causes guanylate cyclases (GCs) to synthesize cGMP leading to the re-opening of the cGMP-gated ion channels and a return to the dark circulating current (Fig. 6)<sup>54</sup>.

Traditionally, the visual cycle has been studied in the rod OS and RPE, but cones are thought to have a unique visual cycle<sup>55</sup>. Cones are specialized to function in day light, when the amount of 11-*cis*-retinal increases due to constant light<sup>55</sup>. The cone cycle is believed to take place in the photoreceptors and the Müller glia cells<sup>55</sup>. The cones regenerate 11-*cis*-retinal in the Müller cells from 11-*cis*-retinol, which is formed from all-*trans*-retinol in the photoreceptors<sup>55,56</sup>. All-*trans*-retinol is transported to the cone inner segment and oxidized to 11-*cis*-retinal to form visual pigment<sup>55,56</sup>.





**Figure 6.** Two important pathways, the retinoid cycle (**A**) and the phototransduction cascade (**B**), are involved in visual processing. **A**) The retinoid cycle recycles the light-absorbing photon and converts 11-*cis*-retinal bound to opsin (Rho) into all-*trans*-retinal. All-*trans*-retinal is reduced by retinol dehydrogenase (RDH) to all-*trans*-retinol. All-*trans*-retinol is exported to the RPE and converted to 11-*cis*-retinol by RPE65, and oxidized to 11-*cis*-retinal by NAD and a *cis*-specific retinol dehydrogenase (*cis*-RDH). 11-*cis*-retinal is exported back to the OS to opsin. (CRALBP cellular retinaldehyde-binding protein, CRBP cellular retinol-binding protein, IRBP interphotoreceptor matrix retinoidbinding protein). **B**) The phototransduction cascade converts light stimuli to electrical signal. Activated opsin (R\*) activates transducin (G) to G\* which in turn activates phosphodiesterase (PDE) to PDE\*\*. PDE\*\* hydrolyzes cGMP, reducing its cytoplasmic concentration. This results in closure of cGMP-gated CNG-channels. This causes hyperpolarization of the photoreceptors leading to signals sent to the downstream neurons. Mutations in several genes that function in the visual cycle have been associated with retinal degenerations. Mutations in genes important for visual pathways have been associated with abnormal vision including *RPE65*, *RHO*, and genes such as *CNGB1* and *CNGB3*, important in the CNG channel formation. The figure is modified from (Nawrot et al. 2006<sup>57</sup> and Pugh 1999<sup>50</sup>).



## **1.6 Canine vision disorders**

Different breed clubs in many countries have obligatory ophthalmoscopy screenings for dogs used for breeding. This helps to identify, follow and study vision disorders in dogs. Finland uses the European College of Veterinary Ophthalmology (ECVO) scheme ([www.ecvo.org](http://www.ecvo.org)), which has been implemented in ten European countries. Over 20,000 dogs are eye examined yearly in Finland by ECVO panelists, which is more than elsewhere in Scandinavia (personal communication with DVM Kaisa Wickström). The examined dog receives a certificate, which is valid from one to three years depending on the breed's eye scheme (ECVO). The results are stored in an open access database maintained by the Finnish Kennel Club ([jalostus.kennelliitto.fi/](http://jalostus.kennelliitto.fi/)). This publicly available database, although not fully accurate, is a valuable resource for research as affected dogs can be found from the database and the owners can be contacted to obtain samples and additional information on the dogs and the diseases. Furthermore, there are several private research-orientated ECVO panelists interested in collaborating with genetic research and ready prepared to provide samples and essential clinical data for the genetic studies.

Dogs are diagnosed with several inherited eye diseases affecting different parts of the eye, such as the lens, retina, ICA, cornea, vitreous etc. The Online Mendelian Inheritance in Animal database (OMIA) lists 57 different hereditary eye diseases in dogs<sup>14</sup>, with cataract being the most frequent ocular disease diagnosed in dog<sup>58</sup>, followed by retinal degenerations and glaucoma, which affect a large number of pure bred dogs<sup>59</sup>.

Many eye diseases are recessively inherited, although dominant, X-linked and complex mode of inheritance have also been described<sup>14</sup>. Inbreeding is likely to have enriched recessive mutations, and as many eye diseases are late-onset they may not have been selected against, since they are only diagnosed after the dog has been used for breeding.

The following chapters will review two neurodegenerative eye conditions, relevant for the study, retinal degeneration and glaucoma, in more detail.

## **1.7 Canine degenerative retinal diseases**

Many different breeds are affected with different types of degenerative retinal diseases affecting different parts of the retina (OMIA)<sup>14</sup>. The spontaneous canine retinal diseases are probably one of the best-described and studied diseases, both clinically and genetically. Canine retinal degenerations form a heterogeneous group of disorders with breed-specific phenotypes observed even among those diseases that arise from

the same mutation (Table 1). On the other hand, similar phenotypes caused by distinct mutations in different genes are diagnosed, in various breeds<sup>17</sup>. For example, in the Golden Retriever there are at least three genetically different types of retinal degeneration caused by different genes<sup>61-63</sup>. While the involved genes vary, the apoptotic cell death appears as a common pathway in retinal diseases<sup>64</sup>, resulting from cell death pathway activation, which is mutation specific, and leading to the degeneration and death of photoreceptors and eventual blindness<sup>65</sup>. Many retinal diseases remain to be clinically and genetically characterized across breeds.

Retinal degenerations can be classified in different ways, such as according to age onset, the affected part of the retina or whether the disease is progressive or stationary. In addition, other developmental disorders may affect the retina, such as microphthalmia<sup>21</sup> and Collie eye anomaly (CEA)<sup>66</sup>.

The most typical type of canine retinal degeneration is a progressive retinal atrophy (PRA) that is primary a rod degeneration. Most PRAs are recessively inherited although some dominant<sup>60</sup> and X-linked<sup>67-69</sup> forms are also known. In PRA, the light sensitive rod photoreceptor cells degenerate first, resulting in a visual impairment in dim light with night blindness<sup>59</sup>. Commonly, as the disease progresses, the cone cells become also affected leading to a complete loss of vision<sup>59</sup>. However, the rate of progression varies significantly in individual dogs and in different breeds, suggesting modulatory genetic or environmental factors<sup>59</sup>.

Ophthalmoscopically the clinical signs in PRA include bilateral tapetal hypo-reflectivity, and later hyper-reflectivity, which is caused by retinal thinning. There is also retinal vascular attenuation, sometimes pigmentary changes and pale optic disc<sup>59</sup>. Clinically PRA is diagnosed on the basis of the specific fundus changes and an abnormal electroretinogram (ERG). If available, optical coherence tomography (OCT) is also used. ERG detects the electric responses from the retina while the OCT is a three-dimensional visualization method. Clinically it is challenging to distinguish types of PRAs that are genetically different as the clinical signs in the retina and ERG responses are usually similar. This highlights the value of genetic research and genetic testing for providing differential diagnoses.

In addition to progressive retinal diseases, stationary types of retinal degenerations and primary cone diseases are also diagnosed in dogs (Tables 4 and 5).

### **1.7.1 Early-Onset Progressive Retinal Atrophy (PRA)**

Early-onset retinal degenerations manifest between 2-6 weeks, during the postnatal differentiation period<sup>26</sup>. The retinal development is abnormal or

arrested and the disease progression is usually quite rapid leading to end-stage disease (Table 1)<sup>26</sup>.

Irish Setters are affected with an early-onset degeneration named RCD1, where the rod photoreceptor development is arrested, leading to the loss of almost all rod cells by 5 months of age<sup>26</sup>. The affected dogs have less rod outer segment material, short inner segments and fewer visual cells<sup>70</sup>. As the disease progresses cone cells with a broad inner segment and a short and abnormal outer segment are detected<sup>70</sup>. Aguirre et al. showed that affected dogs have abnormal cyclic GMP metabolism and that the deficiency in cyclic GMP-phosphodiesterase activity impairs their ability to hydrolyze cyclic GMP in the retina leading to accumulation of GMP-phosphodiesterase in the retina<sup>70,71</sup>. A mutation in the *rod cGMP-specific 3',5'-cyclic phosphodiesterase subunit beta (PDE6B)* has been found in the affected dogs<sup>72</sup>.

*PDE6B* encodes the  $\beta$  subunit of rod photoreceptor cyclic guanosine monophosphate (cGMP)-phosphodiesterase (PDE), which is a vital enzyme of the visual phototransduction cascade. The PDE6 enzyme consists of an  $\alpha$  and  $\beta$  subunit, and two  $\gamma$  subunits. The activated cGMP-PDE complex leads to a reduced intracellular concentration of cGMP and the closure of the cGMP-gated cation channels on rod cells leading to a neural response to light stimulus<sup>73,74</sup>.

In addition to Irish Setters, other mutations in *PDE6B* have been found in the Sloughi<sup>75</sup> and American Staffordshire Terrier<sup>76</sup>. The Sloughi have a later onset disease with visual abnormalities appearing at around 2 years of age<sup>75</sup>. In contrast, the retinal disease in the American Staffordshire Terrier is very similar to the disease seen in Irish Setters<sup>76,77</sup>. The *PDE6B* gene has also been associated with retinal degeneration in mice<sup>78</sup> and humans<sup>79</sup> with a similar phenotype to dogs. The mice are affected with a complete loss of rod cells by postnatal day 20 with an increased level of cGMP<sup>78</sup>.

Another guanosine monophosphate (cGMP)-phosphodiesterase (PDE) related mutation has been found in the  $\alpha$ -subunit. A mutation in the *phosphodiesterase 6A, cGMP-specific, rod, alpha (PDE6A)*, termed RCD3 has been found in the Cardigan Welsh Corgi<sup>80</sup>. The affected dogs have clinical signs typical of an early-onset PRA starting at 6 to 16 weeks and leading to blindness at one year of age<sup>81</sup>. Some dogs may have limited central vision until the age of 3 to 4 years<sup>81</sup>.

A more detailed clinical study was performed by Tuntivanich et al. who showed that the affected dogs have abnormal rod cell outer segment development and reduced light adapted responses<sup>82</sup>. The cone cells are relative well preserved<sup>82</sup>. The retinas lack the PDE6A, but also other PDE6 complex subunits leading to a lack of PDE6 enzymatic activity<sup>82</sup>. In addition to dogs the *PDE6A* mutation has been reported to cause retinal degeneration in mice with increased level of cGMP<sup>83</sup> and in humans with a

severe, early-onset retinal disease<sup>84-89</sup>. This indicates that the PDE6A is a vital part of the PDE6 enzyme.

Another type of RCD, termed RCD2, has been described in the Rough and Smooth Collies<sup>90</sup>. The affected dogs have night blindness at the age of 6 weeks and ERG-confirmed retinal dysfunction already around 16 days of age<sup>90</sup>. The photoreceptor outer segments are underdeveloped and disappear completely during disease progression<sup>90</sup>. The disease affects both rod and cone cells during the postnatal development period, although the cone cells degenerate more slowly<sup>90</sup>. An unusual observation compared to other PRAs, is the accumulation of lamellar bodies in the RPE, hypothesized to be of mitochondrial origin, at a very early age<sup>91</sup>. RCD2 is caused by a repeat sequence insertion in the *retinal degeneration 3 (RD3)* gene leading to an extended, abnormal reading frame<sup>92</sup>.

The *RD3* gene is highly expressed in photoreceptor cells and *rd3* defective mice show a rapid loss of these cells and outer nuclear layers during the second postnatal week<sup>93,94</sup>. In humans multiple different mutations have been found in the *RD3* gene causing early-onset, severe RP and Leber congenital amaurosis (LCA)<sup>95</sup>.

An RCD1- and RCD3-like disease has been described in the Norwegian Elkhound with initial night blindness and total vision loss at 12 to 18 months of age<sup>96</sup>. Both the structural and functional postnatal development of photoreceptor cells appear abnormal<sup>96</sup>. Although, the inner- and outer segments of photoreceptors develop normally, the synaptical terminals of rods and cones remain underdeveloped and result in incoordinated function<sup>96</sup>. A SINE insertion was found in the *serine/threonine kinase 38-like (STK38L)* gene in the affected NEs<sup>97</sup>. The gene belongs to a nodal-related subclass of AGC protein kinases, which are involved in cell division control and cell morphogenesis also in neurons<sup>98</sup>. Berta et al. studied *STK38L* in the canine retina and concluded that defective photoreceptors undergo cell death or proliferation generating a new class of hybrid rod/S-cone photoreceptors suggesting a role for *STK38L* in the control of cell division and photoreceptor morphogenesis<sup>99</sup>.

Another type of early-onset PRA has been described in the Miniature Schnauzer (MS). Although the clinical signs become visible at dogs around 4 years of age, histologically the disease can be diagnosed already at the end of the postnatal developmental period and affects both rod and cone cells<sup>100</sup>. Originally the disease was thought to be caused by a mutation in the *phosducin (PDC)* but this was excluded by further studies<sup>100</sup>. The MS PRA has been named Type-A PRA and a gene test is offered for an unpublished mutation, to test dogs ([www.optigen.com](http://www.optigen.com)). However, Type-A disease only causes a minority of the cases in the Miniature Schnauzer and a yet to be identified gene is responsible for the other disease types ([www.optigen.com](http://www.optigen.com)).

**Table 1.** Several breeds are affected with an early-onset PRA.

Gene	Breed	CFA	Mode of inheritance	Phenotype*	Age of diagnosis	Mutation found in humans	Ref
	Miniature Schnauzer		recessive	PRs affected at the end of the postnatal period	congenital		100
<i>PDE6B</i>	Irish Setter, Sloughi, American Staffordshire Terrier	3	recessive	abnormal rods, short IS, fewer visul cells, accumulation of GMP	congenital	✓	72, 75, 76, 93
<i>PDE6A</i>	Cardigan Welsh Corgi	4	recessive	abnormal rod OS and light adapted responses, cones preserved	6-16 weeks	✓	80, 84-89
<i>RD3</i>	Rough and Smooth Collie	7	recessive	OS underdeveloped and disappear completely	16d-6 weeks	✓	92, 95
<i>STK38L</i>	Norwegian Elkhound	27	recessive	abnormal structural, functional postnatal development	12-18 mo		97

\*(PR, photoreceptor)

### 1.7.2 Late-onset PRA

Late-onset PRAs are usually diagnosed in two-to-three-year-old dogs, although variation is quite extensive across breeds (Table 2)<sup>17</sup>. The retinal changes are diagnosed after the retinal development and maturation period, and the disease progression is slower than in early-onset disease<sup>17</sup>.

One of the most common mutations causing late-onset PRA was found in the *progressive rod cone degeneration (PRCD)* gene<sup>63</sup>. In *PRCD*-PRA, the normal postnatal retinal development is followed by a significant reduction in rod outer segment (ROS) renewal, and the development of early disease in the rod outer segments. A complete photoreceptor loss occurs late in the disease<sup>101,102</sup>.

The *PRCD*-PRA was originally characterized in Miniature Poodles<sup>101</sup> and the *PRCD* locus was mapped to CFA9 using linkage analysis<sup>103</sup>. The locus was further narrowed down<sup>103,104</sup> and a recessively inherited

mutation in the *PRCD* gene was identified<sup>63</sup>. Today the *PRCD* mutation has been found from more than 30 breeds ([www.optigen.com/opt9\\_test\\_prd\\_pra.html](http://www.optigen.com/opt9_test_prd_pra.html)).

*PRCD* is important in the maintenance of the rod photoreceptor function and structure and it is expressed in the photoreceptor disc and in the outer segments of both rod and cone cells<sup>105</sup>. Three mutations in *PRCD* have also been found in human patients with retinitis pigmentosa<sup>63,106,107</sup>. The patients are affected with a recessive RP with typical clinical signs of the disease, including vessel attenuation and pigmentation<sup>106</sup>, similar to the canine *PRCD* model<sup>63</sup>.

Another recessively inherited late-onset PRA has been diagnosed in the Schapendoes breed<sup>108</sup>. The affected dogs show initial night blindness at 2-5 years of age followed by a loss of day vision as the disease progresses<sup>108</sup>. Histologically the outer retina with the photoreceptor layer and the outer nuclear layer are affected<sup>108</sup>. The inner retina shows reduced inner nuclear and inner plexiform layers, whereas the ganglion cell layer appears normal<sup>108</sup>. The causative mutation was found from the *coiled-coil domain containing 66 (CCDC66)* gene<sup>109</sup>.

*CCDC66* expression in the retina is prominent in the inner segments of photoreceptor cells and at a lower level in the external plexiform and ganglion cell layers<sup>109</sup>. *CCDC66* has been shown to cause retinal degeneration in a mouse model<sup>110</sup>. The *Ccdc66* mutant mice show degeneration of photoreceptors already at 13 days of age, followed by a slow, progressive retinal degeneration<sup>110</sup>. In the wild-type mouse, the *Ccdc66* protein is present at highest levels after birth, followed by a decline until adulthood, suggesting a crucial role in the early development<sup>110</sup>. This supports the proposed role of *CCDC66* gene as a causative factor for early, slow progressive rod–cone dysplasia<sup>110</sup>.

Other late-onset, recessive PRA mutations have been found in Golden Retrievers. These include mutations in *PRCD*, in a *solute carrier anion exchanger (SLC4A3)*<sup>61</sup> and *tetratricopeptide repeat domain 8 (TTC8)*<sup>62</sup> genes. *SLC4A3* explains the majority of PRA cases while the *PRCD* and *TTC8* mutations account for 39 % of the affected dogs<sup>62</sup>.

The *SLC4A3* gene mediates the intracellular pH via  $\text{Cl}^-/\text{HCO}_3^-$  exchange in cell membranes<sup>111</sup>. This anion exchanger is expressed in the Müller and horizontal cells of the retina<sup>112</sup> and in the retinal pigment epithelium (RPE) cells<sup>113</sup>. *SLC4A3* also contributes to the removal of photoreceptor-generated  $\text{CO}_2$  waste, which contributes to the maintenance of the acid-balance in the inner retina<sup>114</sup>. A defective *SLC4A3* causes deregulation in pH mechanism and a selective inner retina defect followed by photoreceptor degeneration in mice<sup>114</sup>.

The *TTC8* gene is part of the BBSome complex, which is composed of seven Bardet–Biedl syndrome proteins, functions at the ciliary membrane and is thought to play a role in ciliogenesis<sup>115</sup>. *TTC8* is expressed in the

connecting cilium in the retina<sup>115</sup>. It has been associated with Bardet-Biedl syndrome and non-syndromic RP in humans<sup>116,117</sup>.

Although several late-onset PRAs are diagnosed in Golden Retrievers, it has not been shown which part of the retina is primarily affected in PRAs caused by *SLC4A3* and *TTC8*. The symptoms begin with night blindness indicating a rod mediated disease. However, it is possible that the primary degeneration begins from some other part of the retina and rods are secondarily degenerated. The *SLC4A3* and *TTC8* genes explain a majority of the PRA cases, but neither are known to be expressed in rod cells, which may indicate that the primary tissue is indeed some other layer of the retina.

In addition to Golden Retrievers, Gordon and Irish Setters are affected with a recessive, very late-onset PRA in which the average age of diagnosis is around 10 years<sup>118,119</sup>. The affected Gordons with the late-onset disease present initial night blindness prior to extensive visual impairment, which indicates a rod-mediated degeneration although this has not been histologically confirmed<sup>118</sup>.

The causative mutation has been mapped to *chromosome 2 open reading frame 71 (C2orf71)* gene<sup>118</sup>. *C2orf71* is highly expressed in the eye and is localized to photoreceptors, specifically the outer segment and connecting cilium<sup>120</sup>. Mutations in *C2orf71* in human RP patients have also been identified<sup>120-123</sup>. In addition, Irish Red and White Setters can also be diagnosed with an early-onset PRA caused by the *PDE6B* gene<sup>72</sup>.

More recently a SINE insertion in a *family with sequence similarity 161, member A (FAM161A)* gene was suggested to be the cause of the late-onset PRA in Tibetan Spaniels and Tibetan Terriers<sup>124</sup>. Both breeds are affected with a typical type of PRA<sup>124,125</sup>. The age of onset is around 4-5 years, the disease is recessively inherited and bilateral with typical retinal lesions diagnosed<sup>124,125</sup>. However, the disease progresses rapidly leading to the loss of vision in a couple of years from the initial diagnosis<sup>124,125</sup>.

*FAM161A* is expressed in several tissues including the retina<sup>126</sup> and it has been located in the inner photoreceptor segment, connecting cilium and in the centriole of photoreceptor cells<sup>127,128</sup>. In humans mutations in *FAM161A* have been associated with recessive RP<sup>126,129-131</sup> and in a *Fam161a* mouse model, the mice present a progressive loss of photoreceptors and severely impaired vision<sup>132</sup>. The microglia show alterations to their morphology and have migrated to the outer retina<sup>132</sup>. The affected mice show accumulation of rhodopsin in the photoreceptor inner segment<sup>132</sup>. However, the retinal architecture is fairly well preserved, although the connecting cilium length and the spreading of the microtubules are reduced<sup>132</sup>.

A *S-antigen (SAG)* mutation was also recently associated with PRA in Basenjis with initial visual loss in dim light, which gradually progressed to total blindness<sup>132</sup>. The initial visual loss affects the peripheral visual field,

but the affected dogs retain adequate forward daylight vision for many years, sometimes for their entire natural life<sup>133</sup>.

The *S-antigen* or *arrestin* gene is expressed in the rod outer segment and belongs to a family of inhibitory proteins that bind tyrosine-phosphorylated receptors to block their interaction with specific G-proteins to terminate a signal chain<sup>134</sup>. In the retina, arrestin binds to activated rhodopsin in the rod outer segments<sup>134</sup> to slow G-protein binding and quenches rhodopsin activity<sup>135</sup>. In humans, mutations in this gene have been associated with Oguchi disease, a rare form of autosomal recessive stationary night blindness (OMIM 613411, 258100) and RP<sup>136-38</sup>.

In addition to several recessively inherited retinal degenerations, a dominant form of PRA has been diagnosed in the English Mastiff<sup>60</sup> and its relative breed, Bull Mastiff<sup>139</sup>. The clinical features of the Mastiff PRA include a normal ERG function until the age of three to six months<sup>60</sup>. After this, the ERG responses become abnormal and the disease progression is detected as a focal photoreceptor degeneration<sup>60</sup>. A unique characteristic of this phenotype is a dose-response between light exposure and the early alterations in the retina. The higher light dose causes a more rapid loss of retinal neurons<sup>60</sup>.

The causative mutation has been found in the *rhodopsin* (*RHO*) gene<sup>60</sup>. Rhodopsin functions as part of the visual cycle where 11-*cis*-retinal is converted into all-*trans*-retinal and facilitates the binding of transducin to rhodopsin, subsequently generating an electrical signal via channels gated by cyclic guanosine monophosphate<sup>140,141</sup>. Rhodopsin is light-sensitive and light induces the *cis*-to-*trans* conversion, which further increases the amount of active rhodopsin<sup>140,141</sup>. Active rhodopsin acts as a guanine nucleotide exchange factor for transducing<sup>140,141</sup>.

In humans, over 100 mutations have been found in *RHO*<sup>142</sup>. The *RHO* mutations cause two distinct phenotypes, an early-onset disorder with a rapid loss of rod cells across the retina, and the second phenotype with normal rod vision early in the life with degeneration slowly spreading from focal retinal regions<sup>142-144</sup>. Abnormally slow rod recovery after bright light exposure is associated with the phenotype<sup>143,144</sup>.

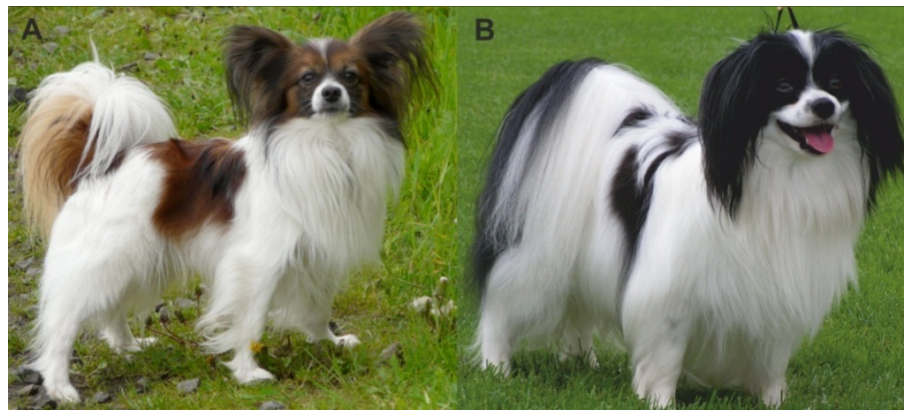
### 1.7.3 Late-onset PRA in Papillon and Phalène breeds

The Papillon breed is affected with an autosomal recessive, late-onset PRA with a mean onset age of 5.6 years<sup>145</sup>. However, variability is detected in the onset possibly due to genetic heterogeneity or environmental factors<sup>146</sup>. Based on the electroretinogram (ERG) recordings, affected dogs have a primary loss of rod photoreceptor cells, followed by the subsequent loss of cone cell function<sup>147,148</sup> with thinning of the outer nuclear layer with age, tapetal reflectivity and retinal vascular attenuation followed by pigment migration<sup>146</sup>. The first clinical sign is the



loss of night vision<sup>145</sup>. The disease progress very slowly and the affected dogs seem to be visually normal throughout their life, as the cone function is fairly well preserved<sup>147,148</sup>.

The American Kennel Club registers only a single Papillon breed, but in Europe the breed is separated into two different breeds, Papillon and Phalène (Fig. 7). The separation is based on the ear morphology, with Papillons having bricked ears while Phalènes have more low set ears (Fig. 7). The littermates are registered on the basis on their ears and because of this the breed is expected to have a similar genetic background. Moreover, since affected dogs with a similar PRA have been found in both forms, it is reasonable to hypothesize that the genetic cause of PRA is also shared. The genetic cause, however, has remained unknown until this study.



**Figure 7.** Papillon (A) and Phalène (B) breeds are separated only by their ear morphology in Europe. Both forms suffer from a PRA which genetic cause was unknown prior to this study.

**Table 2.** Several late-onset PRAs are caused by various genes in dogs.

Gene	Breed	CFA	Mode of inheritance	Phenotype*	Age of diagnosis	Mutation found in humans	Ref
<i>RHO</i>	English and Bull Mastiff	20	dominant	PR loss, dose-response to light	3-6 mo	✓	60, 142
<i>PRCR</i>	Multiple breeds	9	recessive	ROS reduction, PR loss at late stage	variable	✓	63
<i>CCDC66</i>	Schapendoes	20	recessive	NB, affects PRs and ONL	2-5 y		109
<i>SLC4A3</i>	Golden Retriever	37	recessive	NB indicates rod disease	variable		61
<i>C2orf71</i>	Gordon, Irish Setter	17	recessive	NB	10 y	✓	118, 120-123
<i>CNGB1</i>	Papillon, Phalène	2	recessive	NB, PR loss, cone preserved late stage, thinning of ONL	5-6 y	✓	I, 84, 351-354
<i>SAG</i>	Basenji	25	recessive	NB, daylight vision retained		✓	133, 136-138
<i>MERTK</i>	Swedish Vallhund	17	recessive	three stages, origin from rods or RPE	variable	✓	IV, 332, 334-341, 355, 357, 358
<i>TTC8</i>	Golden Retriever	8	recessive	NB indicates rod disease	variable	✓	62, 116, 117
<i>FAM161A</i>	Tibetan Spaniel, Terrier	10	recessive	typical signs of PRA	4-5 y	✓	124, 126, 129-131

\*(PR, photoreceptor, NB, night blindness)

#### 1.7.4 X-linked PRAs

An X-linked, late-onset PRA (XLPRA1) has been described in the Siberian Husky and Samoyed breeds (Table 3) with a deletion in the *retinitis pigmentosa GTPase regulator (RPGR)* gene<sup>68</sup>. The affected males have progressive loss of rods with a secondary loss of cone cells. The age of onset is at young adulthood. Female carriers have focal areas of rod degeneration due to random X-chromosome inactivation<sup>68</sup>.

Another X-linked PRA, but with early-onset, termed XLPRA2 has been diagnosed in mongrel dogs (Table 3)<sup>67</sup>. XLPRA2 is caused by a different deletion in the *RPGR* gene, which severely affects the early stages of the photoreceptor development<sup>67</sup>. XLPRA2 is very severe and manifests during the retinal development. The first clinical signs are detected by 5-6 weeks and even in carriers the retina fails to develop normally<sup>67</sup>.

*RPGR* is highly expressed in the connecting cilium and in the outer segment of photoreceptors<sup>149,150</sup>. It interacts with intraflagellar transport proteins and is hypothesized to function in the cellular trafficking in the connecting cilium<sup>151</sup>.

In humans, one of the most severe forms of RP is caused by mutations in *RPGR*<sup>152-156</sup>. The earliest clinical signs in males include night blindness with onset at the first decade of life. The disease progresses to a reduction in the visual fields and acuity and to blindness by 40 years of age<sup>157</sup>. Similarly to dogs, human female carrier patients exhibit a range of phenotypes that can vary from asymptomatic to a severe retinal disease<sup>158</sup>.

An *RPGR*-independent X-linked PRA, XLPRA3, has been described in Border Collies (Table 3)<sup>69</sup>. The initial clinical signs are detected around 2-3 years of age as a loss of peripheral vision, night blindness, pigmentary changes and attenuated retinal vessels. The degenerative areas are initially focal but evolve towards a generalized degeneration. The mode of inheritance was confirmed as X-linked though the causative gene has not been found<sup>69</sup>.

**Table 3.** X-linked PRA affect the males more severely than carrier females.

Gene	Breed	CFA	Mode of inheritance	Phenotype	Age of diagnosis	Mutation found in humans	Ref
<i>RPGR</i>	Siberian Husky, Samoyed	X	recessive	loss of rods and cones (males), focal rod regeneration (females)	young adulthood	✓	68, 152-156
<i>RPGR</i>	Mongrel	X	recessive	early developmental stages of the PR development	5-6 weeks	✓	67, 152-156
-	Border Collie	X	recessive	loss of peripheral vision, NB, pigmentary changes, evolve to generalized degeneration	2-3 y		69

### 1.7.5 Cone-rod degenerations

In addition to the typical rod degenerations primary cone-rod degenerations (CRD) have been diagnosed in dogs (Table 4). In CRD, cone cells degenerate first, followed by rod cells<sup>21</sup>. As cone cells function in bright light, the primary clinical signs involve day blindness<sup>21</sup>.

One type of cone-rod degeneration (*crd3*) is diagnosed in the Glen of Imaal Terrier<sup>159,160</sup>. The affected dogs show complete loss of retinal layers and a lack of rhodopsin signal<sup>159,160</sup>. Photoreceptor cells are shortened and disorganized and the ONL is thinner<sup>159,160</sup>. The disease is diagnosed at about three years of age and progresses to an end-stage disease in a few years<sup>159,160</sup>.

A deletion that removes two exons in the *ADAM metalloproteinase domain 9 (ADAM9)* gene has been identified as a cause of *crd3*<sup>159,160</sup>. *ADAM9* belongs to a group of metalloproteases with multiple functional domains such as an N-terminal pro-domain, a metalloprotease domain, a disintegrin motif and a cysteine-rich region, an epidermal-growth factor (EGF) repeat, a trans-membrane domain, and a cytoplasmic tail with potential SH3 ligand domains<sup>161</sup>. The gene functions in cone cells and in the regions of high photoreceptor density<sup>162</sup>.

*Adam9*<sup>-/-</sup> mice show a progressive degeneration of rods and cones<sup>162</sup>. Their retinas show an abnormal gap between the distal tips of the photoreceptor outer segment and the RPE apical membrane<sup>162</sup>. In humans, *ADAM9* mutations have been associated with CRD, termed CORD9, with a childhood-onset loss of central and peripheral vision<sup>162,163</sup>.

Another canine CRD model has been described in the Standard Wire-Haired Dachshund with early-onset CRD<sup>164,165</sup>. The age of onset varies from 10 months to 3 years of age with a late stage disease at 6 years. Clinical characteristics include cellophane-like increase in the tapetal sheen and pigment migration in the non-tapetal fundus<sup>164,165</sup>. The ERG indicates reduced cone-derived responses already at 5 weeks of age, while the rod-derived responses are less affected<sup>164,165</sup>.

The causative mutation has been found in the *nephronophthisis 4* (*NPHP4*) gene<sup>166,167</sup>. The gene is commonly associated with nephronophthisis, a kidney disease<sup>168</sup> and nephronophthisis with retinitis pigmentosa, Senior-Løken syndrome<sup>168</sup>. However, *NPHP4*-deficient mice also develop severe photoreceptor degeneration and reduced rod and cone ERG responses by 9 weeks of age<sup>169</sup>. *NPHP4* has been reported to interact with *nephronophthisis 1* (*NPHP1*), *RP GTPase regulator interacting protein 1* (*RPGRIP1*), *RP GTPase regulator interacting protein 1 like* (*RPGRIP1L*) and *retinitis pigmentosa GTPase regulator* (*RPGR*)<sup>150,168,170,171</sup>.

A mutation in *retinitis pigmentosa GTPase regulator-interacting protein* (*RPGRIP1*) gene, which interacts with *NPHP4*, has been found in the Miniature Longhaired Dachshund<sup>172</sup>. The affected dogs are diagnosed with abnormal ERG at 6 weeks and blindness by the age of 2 years<sup>173</sup>. The disease primarily affects cone cells<sup>173</sup>. The disease is termed as CORD1<sup>174</sup>. A fully segregating 44-bp insertion in the 3'UTR of the *RPGRIP1* gene was originally found in a research colony<sup>172</sup>, however the mutation was also present in a normal pet population without ophthalmoscopical findings<sup>175</sup>. A further study of the phenotype led to the identification of another locus co-segregating with the *RPGRIP1* mutation<sup>176</sup>. This indicates that the disease may be digenic and the *RPGRIP1* mutation alone does not cause the disease phenotype<sup>176</sup>.

*RPGRIP1* is expressed in amacrine neurons, photoreceptors and in many other eye tissues<sup>149</sup>. Studies in knockout mice indicate that *RPGRIP1* is required for the morphogenesis of the outer segment (OS) discs, particularly in rods<sup>177</sup>. *RPGRIP1* belongs to the cilia protein network and is part of various protein complexes in the retina, including *RPGR*<sup>149</sup>, *NPHP4*<sup>170</sup> and *RanB*<sup>178</sup>. In humans mutations in *RPGRIP1* causes Leber Congenital Amanirosis<sup>179</sup> and recessive retinal dystrophy<sup>180</sup>.

**Table 4.** Genetically distinct cone degenerations are diagnosed in several breeds.

Gene	Breed	CFA	Mode of inheritance	Phenotype*	Age of diagnosis	Mutation found in humans	Ref
<i>RPGRIP1</i>	Miniature Longhaired Dachshund	15	recessive	abnormal ERG, blindness, cones primarily degenerated	6 weeks-2y	✓	172, 179, 180
<i>NPHP4</i>	Standard Wirehaired Dachshund	5	recessive	cellophane-like increase in tapetal sheen, pigment migration in the non-tapetal fundus, reduced cone responses, rods less affected	10 mo-3y	✓	166, 168
<i>ADAM9</i>	Glen of Imaal Terrier	16	recessive	complete loss of retinal layers, lack of rhodopsin signal, PR shortened, disorganized, thinner ONL	3y	✓	159, 162, 163

\*(PR, photoreceptor)

### 1.7.6 Other retinal degenerations

PRAs and cone-rod dystrophies are progressive and eventually lead to complete blindness. However, not all PRAs are progressive (Table 5). A retinal dystrophy has been described in the Briard dog, including congenital night blindness and partial day blindness observed already in 5-6 week-old puppies, concurrently with a normal fundus appearance<sup>181</sup>. There is little change in fundus appearance until affected dogs are 3-4 years old<sup>182</sup>, when subtle abnormalities can be detected such as whitish multifocal spots mainly in the tapetal area. ERGs indicate defects in the phototransduction process, most typically with non-recordable ERG rod responses and severely reduced cone responses<sup>183</sup>. Photoreceptors appear mainly normal histologically early in the disease process, while there is an accumulation of lipoidal material in the RPE<sup>183</sup>. The disease is often called the Briard retinal dystrophy but lately the designation canine Leber congenital amaurosis has been used for the disorder.

A mutation in the *retinal pigment epithelium 65 (RPE65)* gene has been indicated as a cause<sup>184</sup>. *RPE65* is vital for the visual cycle and important

for the regeneration of the light sensitive chromophore 11-*cis*-retinal<sup>185,186</sup>. It has also been shown that in *RPE65* deficient dogs, the rod second order neuron sprouting is increased in the inferior periphery<sup>187</sup>. *RPE65* is expressed in RPE cells<sup>188,189</sup>, and cone cells<sup>190</sup>. The S cone opsin expression is not uniformly reduced as RPE seems to preserve S-cone opsin but not L/M-cone opsin expression<sup>187</sup>.

*RPE65* causes type 2 Leber congenital amaurosis (LCA) in human (OMIM 204100)<sup>191</sup> or an early-onset severe retinal dystrophy<sup>192,193</sup>. Also the existing mice models, both induced and spontaneous, exhibit progressive retinal degeneration similar to the dog and human<sup>194-196</sup>.

A stationary retinal disease, termed canine multifocal retinopathy (cmr), is caused by mutations in the *bestrophin* (*BEST1*) gene. CMR has been described in both English and Bull Mastiffs, and Great Pyrenees (cmr1), Coton de Tulear (cmr2) and Laponian Herders (cmr3)<sup>197-199</sup>. Breed-specific alterations in the mutation positions, including a nonsense transition (c.C73T/p.R25X) in cmr1, a missense change (c.G482A/p.G161D) in cmr2 and a frameshift mutation (c.C1388del/p.P463Fs) resulting in a truncated peptide in cmr3, have been reported. The affected dogs have multifocal areas of retinal elevation with an accumulation of subretinal fluid<sup>198,199</sup>. The disease may be stationary for years, but older dogs may also develop a multifocal outer retinal atrophy<sup>198</sup>.

The bestrophin protein is located in the basolateral plasma membrane of the RPE and it functions as a Ca<sup>2+</sup>-dependent anion channel<sup>200</sup>. It participates in the epithelial transport across the RPE<sup>201,202</sup>.

In humans, over 100 *BEST1* mutations have been associated with several phenotypes, including bestrophinopathies, a best vitelliform macular dystrophy (BVMD), a juvenile-onset macular degeneration, an adult-onset foveomacular vitelliform dystrophy, an autosomal dominant vitreoretinopathopathy, and an autosomal dominant retinitis pigmentosa<sup>203,204</sup>.

Another example of a stationary canine retinopathy, cone degeneration, has been identified in the Alaskan Malamute<sup>205</sup>. The affected dogs develop day blindness and photophobia already at 8-12 weeks after the postnatal retinal development period due to degeneration of cone cells<sup>206</sup>. The rod cells develop and function normally and the affected dogs retain normal night vision<sup>206</sup>.

The cone degeneration in Alaskan Malamutes and in the Miniature Australian Shepherd is caused by a large genomic deletion (0.4 Mb), including all exons of the *cyclic nucleotide gated channel beta 3* (*CNGB3*) gene and large parts of the *copine III* (*CPNE3*) and *cyclic nucleotide binding domain containing 1* (*CNBD1*) genes<sup>207,208</sup>. The deletion carriers have been found also in the Siberian Husky, and in the Alaskan Sled Dog breeds<sup>208</sup>. In addition, a missense mutation in the *CNGB3* gene was found in a day-blind German Shorthaired Pointer<sup>207</sup>.

*CNGB3* encodes the  $\beta$ -subunit of the cyclic nucleotide-gated (CNG) channel that represents the light-activated channels located in the cone photoreceptors<sup>209</sup>. The outer segments of the CNG channels consist of  $\alpha$ - and  $\beta$ -subunits<sup>210</sup>.  $\alpha$ -Subunits can form functional channels by themselves, as the  $\beta$ -subunits cannot<sup>211</sup>. The  $\beta$ -subunits modulate channel properties when co-expressed with  $\alpha$ -subunits, forming a native CNG channel<sup>211</sup>. Both subunits are essential for normal CNG channel function in the cone outer segment plasma membrane and are required for generation of light-evoked electrical photoresponses in the cones<sup>211</sup>. Mutations in the *CNGB3* gene lead to abnormal cone function and loss of cone cells as the CNG channel in the cone cells is not properly formed<sup>212</sup>. However, genetic heterogeneity has been described in day blind Alaskan Malamutes, as not all clinically confirmed cases have the *CNGB3* deletion and a yet unknown mutation causes the disease in these dogs<sup>213</sup>.

In humans, mutations in *CNGB3* cause achromatopsia, which is an autosomal recessive disease characterized by a loss of cone photoreceptors and day-blindness, total colorblindness, and decreased central visual acuity<sup>214</sup>.



**Table 5.** *Examples of other types of retinal degenerations and associated genes and breeds.*

Gene	Breed	CFA	Mode of inheritance	Phenotype	Age of diagnosis	Mutation found in humans	Ref
<i>RPE65</i>	Briard	6	recessive	defect in the phototransduction, accumulation of lipoidal material in RPE	5-6 weeks	✓	181, 192, 193
<i>CNGB3</i>	Alaskan Malamute, and Sled Dog, Miniature Australian Shepherd, Siberian Husky, Shorthaired Pointer	29	recessive	day blindness and photophobia after the postnatal development, cone degeneration, normal vision in dim light	8-12 weeks	✓	207, 208, 214
<i>BEST1</i>	English, Bull Mastiff, Great Pyreness, Coton de Tulear, Lapponian Herder	18	recessive	multifocal areas of retinal elevation with accumulation of subretinal fluid	variable	✓	198, 203, 204

## 1.8 Glaucoma

Together with retinal degeneration and cataract, glaucoma is one of the most common causes of irreversible blindness<sup>215,216</sup>. Glaucoma describes a heterogeneous group of ocular disorders that are characterized by a progressive degeneration of the optic nerve head, optic disk changes, focal or generalized thinning of the neuroretinal rim, leading to neurodegeneration of the retinal ganglion cells (RGC) and a progressive defect in the visual fields (Fig. 3)<sup>29</sup>. Elevated intraocular pressure (IOP), which results from an obstruction of the normal flow of aqueous humor through the anterior chamber and the trabecular meshwork, is considered a strong risk factor<sup>29,217</sup>. In humans, glaucoma is a chronic and slowly progressive disease that may severely damage the optic nerve and the retina before any symptoms are recognized<sup>29</sup>.

Glaucoma can be a primary or secondary disease. It is broadly classified into three groups: primary open-angle (POAG), primary closed angle (PACG), and primary congenital glaucoma (PCG)<sup>218</sup>, in all of which, the IOP may or may not be elevated<sup>29</sup>. Genetics of human glaucoma have been extensively studied and multiple loci have been mapped for all types<sup>219-222</sup>. However, only few genes have been associated mainly with familial glaucoma<sup>219,221,222</sup>. This indicates that glaucoma is a complex disease with both genetics and environment contributing to the disease development.

Similarly to humans, many dog breeds are affected with glaucoma<sup>219,221,222</sup>. Canine glaucoma can be a primary, hereditary condition or secondary to some other primary disease such as uveitis or lens luxation<sup>223</sup>. Both primary open and closed angle glaucomas have been described in several dog breeds<sup>21</sup>. Clinical features resemble largely human glaucoma including the loss and death of the RGCs, optic nerve axonal loss and concurrent optic nerve head cup enlargement with incremental reduction in visual fields and blindness with or without IOP elevation<sup>224</sup>. The disease affected both eyes equally, even though the disease progression differs between the eyes with the onset varying by months or even years<sup>224</sup>.

Despite the existence of both POAG and PACG in many breeds the genetic etiology of glaucoma is almost completely unknown in dogs (Table 6). It is hypothesized that both types of glaucoma are inherited but the mode of inheritance remains unknown. In Siberian Huskies and in Welsh Springer Spaniels, a recessive and a dominant model of inheritance, respectively, have been suggested<sup>225,226</sup>. During this study, a mutation in *ADAMTS10* was described in Beagles with POAG<sup>227</sup> and two polymorphisms in *SRBD1* were suggested in Shiba Inus and Shih Tzus with PACG<sup>228</sup>. Furthermore, two loci have been suggested for PACG in the Basset Hound<sup>229</sup>, although further studies are required to confirm these preliminary associations.

### 1.8.1 Primary open angle glaucoma (POAG)

POAG is the most common form of human glaucoma<sup>220</sup>. It is characterized by changes in the ONH and defects in the visual field, with a normal open anterior chamber and progressive death of RGC<sup>230</sup>. The IOP is frequently increased, but may also be within the normal range<sup>230</sup>.

In humans, the genetic basis of POAG has not been completely established due to its heterogeneous etiology<sup>218,219,231</sup>. *Myocilin* (*MYOC*)<sup>232</sup>, *neurotrophin 4* (*NTF4*)<sup>233</sup>, *optineurin* (*OPTN*)<sup>234</sup> and *WD repeat domain 36* (*WDR36*)<sup>235</sup> and at least 20 genetic loci of which 14 are named (GLC1A-N) have been suggested for human POAG<sup>236,237</sup>. Furthermore, putative associations have been found for loci including genes *ICA1*,

*RAB9BP1*, *SLC2A14/SLC2A3*, *LOXL1*, *CAV1/CAV2*, *TCMO1*, and *CDK2BAS*<sup>238-242</sup>.

Canine POAG has been studied in a research colony of Beagles<sup>243-245</sup>. In canine POAG, the ICA and ciliary cleft are initially open and normal. The pectinate ligament is usually normal but may also be slightly dysplastic<sup>245</sup>. As the disease progresses IOP gradually increases, resulting in the ICA and ciliary cleft structure closure. The cupping of the optic nerve head (ONH) and disappearance of the retinal vessel follow the disease progression<sup>246</sup>.

In the colony Beagles, POAG is an autosomal recessive disease and a mutation in the *ADAM metalloproteinase with thrombospondin type 1 motif, 10 (ADAMTS10)* gene has been identified<sup>227</sup>. *ADAMTS10* interacts with fibrillin-1 and localizes to fibrillin-1 microfibril bundles<sup>247</sup>. Microfibrils maintain the lens in its position via lens ligaments, which are primarily comprised of fibrillin-1<sup>248</sup>. Fibrillin-1 is also expressed in the outflow pathway of the aqueous humor and a defect in the fibrillin-1 may lead to impaired aqueous humor flow<sup>249-251</sup>. Kuchtey et al. demonstrated clinically that the affected Beagles have weakened posterior scleral biochemical properties and significant alterations in collagen solubility before ONH damage<sup>252</sup>. Their microfibril theory suggests that glaucoma could be caused by abnormal microfibril rearrangement<sup>252</sup>. The *ADAMTS10* mutation was further tested in other breeds and pet Beagles, demonstrating that the mutation is limited to the Beagle colony<sup>253</sup>.

### 1.8.2 Primary glaucoma in the Norwegian Elkhound

Norwegian Elkhounds (NE) is a spitz type breed, originating from Norway with grey or black fur. It is a common breed in Finland used for elk hunting (Fig. 8). The breed is affected with POAG<sup>254,255</sup>. The disease in NEs is commonly diagnosed in middle-aged or elderly dogs, when it starts to affect the dog's hunting capabilities<sup>254</sup>. However, it is probable that the actual disease onset is earlier<sup>254</sup>. The affected dogs are often brought to a veterinarian only when the disease has already developed into a late stage disease with a significantly elevated IOP. Early clinical signs in NEs include a slightly elevated IOP with a normal opening of the ciliary cleft<sup>254</sup>. The peripheral vision is commonly affected and as the disease progresses, the vision is further impaired due to the cupping and atrophy of the optic nerve head<sup>254</sup>. At the later stages of the disease, the narrowing or collapse of the ciliary cleft contributes to the elevation of IOP. This may lead to secondary subluxation of the lenses in some cases. The retina is normal until the late stages of the disease. Other clinical signs may include Haab's striae, cataract, and buphthalmos<sup>254</sup>. The genetic cause of the disease was unknown prior to this study.



**Figure 8.** The Norwegian Elkhound is a spitz type breed, which is affected by primary glaucoma. It is common in Finland used to hunt elk.

### 1.8.3 Primary closed angle glaucoma (PACG)

PACG in humans is characterized by the shallow anterior chamber, increased thickness of the lens, complete or partial chamber angle closure, hyperopic refractive error, and short axial length<sup>256</sup>. Elevated IOP is caused by a shallow anterior chamber and the obstruction of the iris-trabecular meshwork in the iridocorneal angle of the eye, which causes blockage of the aqueous humor outflow<sup>257,258</sup>.

Recently three loci were associated with PACG, with separate markers showing significant association after replication on human chromosomes 1, 8 and 11, near genes *pleckstrin homology domain containing, family A member 7* (PLEKHA7), *collagen, type XI, alpha 1* (COL11A1), *protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 1* (PCMTD1) and *suppression of tumorigenicity 18, zinc finger* (ST18), respectively<sup>257</sup>. However, no causative variants have been identified. Association studies also suggest the involvement of several single nucleotide polymorphisms (SNPs) of the *matrix metalloproteinase-9* (MMP-9) gene<sup>259-261</sup>. PACG has also been associated with *membrane type frizzled related protein* (MFRP), *methylenetetrahydrofolate reductase* (MTHFR) and the retinal homeobox gene (*CHX10*)<sup>260,262-264</sup>. These genes are involved in the regulation of axial length and structural remodeling of connective tissue. However, this association has not been replicated and the biological significance of the gene in the development of PACG is still unknown<sup>260,262-264</sup>.

Canine PACG is a result of gradually narrowing ICA due to congenital malformation of the ICA structure, resulting in a complete closure of the ICA angle<sup>265</sup>. The pectinate ligaments are severely dysplastic, with sheets

of tissue with or without flow holes or broad ligament strands<sup>265</sup>. A significant and progressive defect in RGC function is diagnosed which progresses to the retinal photoreceptor cells in the outer retina<sup>266,267</sup>. The loss of RGC correlates with the abrupt and severe elevation of IOP, which is commonly associated with PACG<sup>224</sup>. The abrupt elevation of IOP may be caused by the closure of the ICA and blockage of the aqueous humor flow<sup>224</sup>.

It is hypothesized that female dogs have approximately twice the risk of males for developing PACG, due to gender differences in the morphology of the ICA<sup>268,269</sup>. Another suggested factor is age. PACG is usually diagnosed in elderly dogs after 6 years of age but the initial onset may be earlier as the symptoms become observable only at the later stage of the disease when the ICA has narrowed and IOP is significantly elevated<sup>224</sup>. The earlier primary symptoms may go unnoticed if a detailed examination is not routinely performed to evaluate the ICA, ciliary cleft and pectinate ligament structures<sup>224</sup>.

Some dog breeds are more prone to PACG development, including American Cocker Spaniel<sup>270</sup>, Basset Hound<sup>271</sup>, Boston Terrier<sup>59</sup>, Bouvier des Flandres<sup>38</sup>, Chow Chow<sup>272</sup>, English Cocker Spaniel<sup>273,274</sup>, Flat Coated Retriever<sup>275</sup>, Golden Retriever with iridociliary cysts<sup>276</sup>, Great Dane<sup>277</sup>, Toy and Miniature Poodles<sup>59</sup>, Samoyed<sup>278</sup>, Siberian Husky<sup>59</sup>, Shih Tzu<sup>279</sup> and Welsh Springer Spaniel<sup>225</sup>.

In dogs, however, little is known about the genetic background of PACG. Polymorphisms in the *S1 RNA binding domain 1 (SRBD1)* gene have been associated with PACG in Shiba Inus and Shih Tzus but no functional variant has been found yet<sup>228</sup>. In Basset Hounds, two loci were suggested on canine chromosome 14, containing the *collagen, type I, alpha 2 (COL1A2)* and *paternally expressed 10 (PEG10)* genes and on chromosome 24 with a candidate gene *RAB22A, member RAS oncogene family (RAB22A)*<sup>229</sup>. However the p-values in the associated region did not reach the genome wide significance, and require further studies in larger study cohorts.

#### **1.8.4 Primary congenital glaucoma (PCG)**

PCG is the most common form of childhood glaucoma and is characterized by abnormalities in the anterior chamber angle, elevated IOP, corneal oedema, buphthalmos, corneal enlargement, photophobia, lacrimation, blepharospasm, corneal opacity, and optic atrophy in the late stage disease<sup>280,281</sup>. Ocular abnormalities in PCG cause obstructed aqueous humor outflow. The onset of PCG usually occurs within the first few years of life. The disease is often transmitted in an autosomal-recessive pattern and occurs up to 10 times more frequently in certain

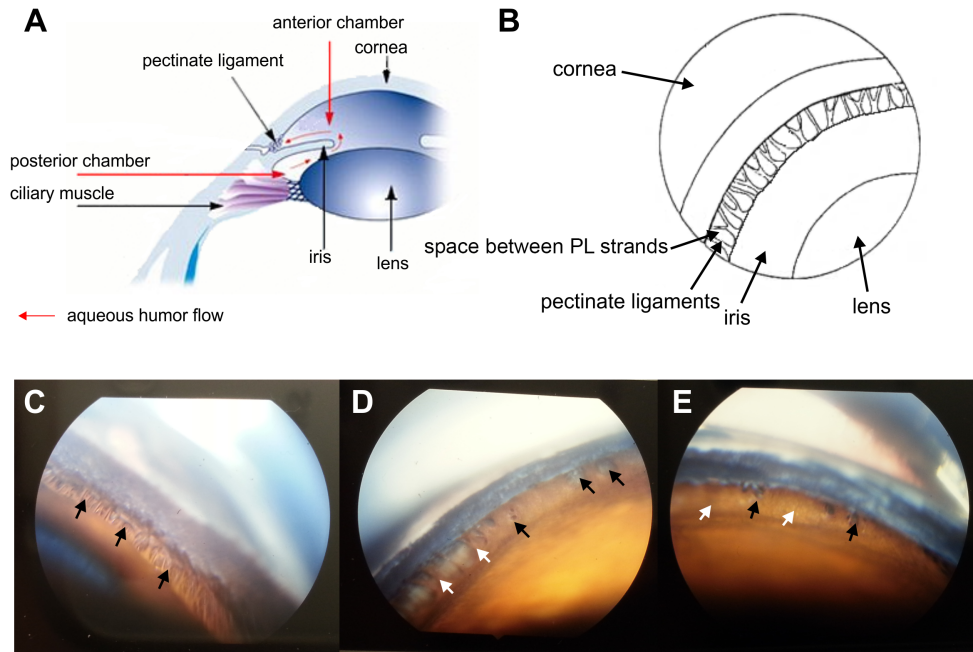
ethnic and religious groups where consanguineous relationships are common<sup>282</sup>.

Three loci (GLC3A-3C)<sup>236,237,283</sup> and two genes *cytochrome P450 1B1 (CYP1B1)*<sup>284</sup> and *latent transforming growth factor beta binding protein 2 (LTBP2)*<sup>285</sup> have been suggested to associate with PCG in humans. In dogs a congenital origin of PACG or POAG cannot be ruled out as the initial disease onset may be congenital, even though clinical abnormalities are only detected in older dogs.

#### 1.8.5 Pectinate ligament dysplasia (PLD)

Pectinate ligament dysplasia (PLD) refers to abnormal pectinate ligament structures and it has been diagnosed in many dog breeds (Table 6). PLD is used to describe congenital ocular abnormalities in the iridocorneal angle (ICA)<sup>275</sup>. In PLD the abnormalities in the pectinate ligaments consist of mesodermal sheet with or without flow holes extending from the base of the iris to the peripheral cornea<sup>38</sup>. The mesodermal sheet may be formed of broader PLs or a solid sheet of tissue instead of normal thin strands, which causes the narrowing of the ICA (Fig. 9)<sup>38</sup>. The ICA is thought to be abnormal if more than 25 % of the ICA circumference is affected<sup>278</sup>. If more than 75% of the ICA is affected, PLD is considered severe<sup>278</sup>. In glaucomatous eyes, PLD is usually severe or the whole ICA is collapsed (Fig. 9). Gender or age probably does not affect the PLD development<sup>225,226, 270,278, 286</sup>.

In addition to PLD abnormalities, the narrowing of the relative width of the opening of the ciliary cleft (RWOC) has been associated with glaucoma<sup>274,278</sup>. Bjerkas et al. have showed that in the English Springer Spaniel both the PLD and the narrowing of the RWOC may contribute to the development of glaucoma that cannot be visualized in conventional gonioscopy<sup>274</sup>.



**Figure 9.** **A)** The pectinate ligament (PL) form the internal boundary of the ICA, between iris and the cornea **B)** A schematic structure of the pectinate ligament through goniolens. **C)** In normal canine eye ligament structures are presented as a pillar of tissue of thin to stout strands of tissue and the aqueous humor flow between the ligament strands (arrows). **D)** In PLD the ligament structure is abnormal with broadened PLs (white arrows) and a solid sheet of tissue with flow holes (black arrows) **E)** In severe PLD, the PLs form a solid sheet of tissue (white arrows) with occasional flow holes (black arrows) blocking the aqueous humor flow. (pictures modified from Holger Funk: [www.shiba-dog.de/shiba-klub/glaukom-en.htm](http://www.shiba-dog.de/shiba-klub/glaukom-en.htm) and [www.rhegedspringerspaniels.com/welshie-health.asp](http://www.rhegedspringerspaniels.com/welshie-health.asp), PLD photos in courtesy of DVM Sanna Elfving).

PLD and abnormal ICA has been described in several breeds including American Cocker Spaniel<sup>270</sup>, Basset Hound<sup>271</sup>, Bouvier des Flandres dogs<sup>38</sup>, English Springer Spaniel<sup>274</sup>, Flat Coated Retriever<sup>275</sup>, Great Dane<sup>277</sup>, Samoyed<sup>278</sup>, Siberian Husky<sup>226</sup>, Shiba Inu<sup>287</sup> and Welsh Springer Spaniel<sup>225</sup>. In many breeds PLD is considered to be hereditary, although the mode of inheritance remains unknown (Table 6)<sup>286</sup>.

Dogs with severe PLD have a significantly increased risk of developing glaucoma. However, other factors have an effect on the glaucoma development as not all dogs with severe PLD develop the disease<sup>38,275,286</sup>. Furthermore, abnormalities in the ICA may be just a clinical marker for a triggering disease process originating deeper in the ICA<sup>275</sup>. A more detailed examination would allow a better evaluation of the ciliary cleft and iridocorneal angle than the commonly used gonioscopy<sup>288</sup>.

**Table 6.** *PLD and glaucoma have been reported in several breeds. However only four loci and one causative gene have been implicated in dogs.*

Gene	Breed	CFA	Mode of Inheritance	Phenotype	Mutation found in humans	Ref
<i>ADAMTS10</i>	Beagle, Norwegian Elkhound	20	recessive	POAG	✓	227, V, 379, 380
	American Cocker Spaniel			PACG, PLD		270
<i>COL1A2, PEG10/RAB22A</i>	Basset Hound	14/24		PACG		271, 229
	Boston Terrier			PACG		59
	Bouvier des Flandres			PACG, PLD		38
	Chow Chow			PACG		272
	Dandie Dinmont Terrier	8	recessive?	PACG, PLD		III
	English Cocker Spaniel			PACG		273
	English Springer Spaniel			PLD		274
	Flat Coated Retriever			PACG, PLD		275
	Golden Retriever			PACG and iridociliary cysts		276
	Great Dane			PACG, PLD		277
	Toy, Miniature Poodle			PACG		59
	Samoyed			PACG, PLD		278
	Siberian Husky		recessive?	PACG, PLD		59
	Shih Tzu	10	recessive	PACG		228, 279
	Shiba Inu	10	recessive	PACG, PLD		228, 279
	Welsh Springer Spaniel		dominant?	PACG, PLD		225



## 1.9 Dog as an animal model for ocular gene therapy

Retinal degenerations and glaucomas have been extensively studied in dogs and several genes have been found. Most canine genes have been the orthologues to those identified in human retinal degenerations and glaucoma<sup>17,289</sup>.

The genetic and pathological findings in dogs have significantly contributed to the understanding of the disease mechanism in humans<sup>290-293</sup>. Novel retinal degeneration genes found in dogs have subsequently been identified in human patients<sup>63,80,92</sup>.

The identification of mutations causing retinal degenerations has provided important canine models for ocular gene therapies prior to translation into human patients<sup>294</sup>. Disease prevention, stabilization and the degenerative process can be efficiently modeled in dogs<sup>294</sup>. The dimensions of the canine eye are similar compared to the human eye, despite smaller vitreous volume and the lack of the foveo-macular region<sup>295</sup>.

Viral vectors, especially recombinant adeno-associated viruses (rAAV) have provided the most promising results in canine gene therapy trials<sup>290-293</sup>. The part targeted most often is the retina. Being anatomically well-defined and readily accessible, the retina offers several advantages. The most typically targeted part of the retina is the RPE or photoreceptor cells<sup>294</sup>. In addition to rAAV mediated therapy, combinatorial treatments including delivery of agents to prevent the loss of retinal cells, such as neuroprotective, prosurvival, or anti-apoptotic factors or antioxidants, sequentially or simultaneously with gene augmentation therapy are under development<sup>294</sup>.

The first successful gene therapy trial in dogs paved the way for a gene therapy for human LCA (*RPE65*). In RPE65 trials, the proof-of-principle studies in young RPE65-deficient dogs showed that rAAV vectors expressing either human or canine RPE65 dramatically improved photoreceptor function<sup>290-293</sup>. The LCA gene therapy has been the first to be implemented in human patients<sup>296-300</sup> and has been shown to improve the patient's visual function, especially in children<sup>301</sup>. However, despite functional recovery, the disease progression continues both in humans and in dogs, implying that the gene replacement therapy alone may not be sufficient and combinatorial treatments may be required<sup>302</sup>.

Other canine gene therapy trials are currently being developed for canine achromatopsia (*CNGB3*) and X-linked PRA (*RPGR*). In the former, a successful canine gene therapy trial has been reported, in which the cone function and associated photopic vision were restored<sup>303</sup>. rAAV vectors with different human red cone opsin promoters were used and the results suggested that the treatment was mutation independent, but promoter and age dependent<sup>303</sup>. Furthermore, the *CNGB3*-achromatopsia study showed that a combined staged therapy of ciliary neurotrophic factor

(CNTF) and rAAV vector successfully restores cone function at older age (14–42 months) when gene augmentation therapy alone cannot<sup>304</sup>. Currently, no human gene therapy has been developed for achromatopsia, but the canine gene therapy trial provides a successful path for translation to human patients.

Similar trials have been developed also for the canine XLPRA caused by the *RPGR* gene. Beltran et al. showed that gene augmentation in both rods and cones with the full-length human *RPGR* driven by two separate promoters, prevented photoreceptor degeneration and preserved the retinal structure and function<sup>305</sup>. Although the canine gene therapy for XLPRA has been successful in a small number of treated animals<sup>305</sup>, human studies could be more complicated due to disease modifiers affecting the large spectrum of *RPGR*-XLPRA phenotypes seen in patients<sup>306</sup>.

## 2 AIMS OF THE STUDY

The overall aim of this study was to identify the genetic causes of two degenerative ocular disorders, retinal degeneration and glaucoma, in five different dog breeds. An increased prevalence of the diseases has been reported in certain breeds, suggesting a genetic predisposition. A collaborative effort combining basic research and clinical studies was embarked on to establish clinically relevant study cohorts in each breed to identify the genetic background of the disorders.

The specific aims of this study were:

- I. To identify the causative mutation for PRA in Papillon and Phalène breeds
- II. To describe the clinical phenotype of primary glaucoma in Dandie Dinmont Terriers and map the disease gene
- III. To clinically characterize the retinopathy phenotype and to find the causative gene in Swedish Vallhunds
- IV. To identify the mutation causing primary glaucoma in Norwegian Elkhounds

## 3 MATERIAL AND METHODS

### 3.1 Study cohorts and pedigrees

DNA samples were collected to the canine DNA repository established by Professor Hannes Lohi at the University of Helsinki and Folkhälsan Research Center, Helsinki, Finland. The DNA bank currently contains over 50,000 DNA samples from 327 different dog breeds and serves as an excellent resource for the genetic research, also in canine vision disorders. Donors were recruited by advertising the research in the website ([www.koirangeenit.fi](http://www.koirangeenit.fi)), contacting the relevant breed clubs and through social media, such as Facebook. An extensive phenotype and genotype database has been established to maintain information about the dogs and owners. All samples were collected from privately owned pet dogs with the owners consent under the permission of the Animal Ethical Committee of County Administrative Board of Southern Finland (ESAVI/6054/04.10.03/2012) and University of Pennsylvania IACUC protocols # 802604 and 803429 (Study III, IV and V).

Large pedigrees were constructed around the affected dogs with a GenoPro genealogy software ([www.genopro.com](http://www.genopro.com)) using the genealogical data available in public canine registries such as the database maintained by the Finnish Kennel Club ([jalostus.kennelliitto.fi](http://jalostus.kennelliitto.fi)) and Hunddata maintained by the Swedish Kennel Club ([hundar.skk.se/hunddata/](http://hundar.skk.se/hunddata/)), or information provided by the owners. All pedigrees can be found in each separate study. EDTA-blood or buccal samples were collected by a veterinarian or trained specialist for different studies as follows:

**Study I:** Samples from 59 Papillons and from 116 Phalènes were collected, including 4 Papillons and 2 Phalènes with clinical signs consistent with PRA and 14 control dogs that were confirmed healthy in an eye examination at the age of over 7 years.

**Study II:** Samples were collected from 33 DDTs diagnosed with glaucoma and 159 control dogs. In addition, 35 samples were collected from DDTs affected with PLD. Control dogs were over 7 years old and had been confirmed to have healthy eyes although the presence of mild PLD (grade 1) was tolerated. The fine mapping study included dogs older than 5 years. In addition, samples were collected from Siberian Huskies with uni- or bilateral glaucoma and increased IOP, Flat Coated Retrievers, and Welsh Terriers presenting mild to severe PLD with a narrowed or closed ICA.

**Studies III and IV:** Samples were collected from 93 retinopathy-affected and 76 unaffected dogs, and from 436 SVs altogether. Controls dogs were

over the age of six years and were confirmed to be free of retinal disease. In study III, ocular histopathologic samples were obtained from 8 SVs, that were euthanized for unrelated reasons, and the eyes donated by the owners. Paraffin sections of normal and affected eyes originating from other canine breeds were provided as control samples by the Anatomic Pathology/Biopsy Service of the University of Pennsylvania School Of Veterinary Medicine and the Comparative Ocular Pathology Laboratory of Wisconsin (COPLOW). In addition, samples from mixed breed colony dogs were obtained from the Retinal Disease Studies Facility at the University of Pennsylvania, School of Veterinary Medicine. In study IV retinal samples were collected from four retinopathy affected dogs, that were euthanized for unrelated causes.

**Study V:** Samples were collected from 16 glaucoma affected, dogs 9 controls and 572 population dogs. The control dogs were eye examined at the age of at least 8 years old and did not show any clinical signs of glaucoma.

In all studies additional DNA and tissue samples from the DNA-bank were utilized to study the genes and variants within the breeds and at the population level.

### **3.2 Genomic DNA and RNA extraction**

Genomic DNA was extracted from EDTA blood or buccal samples, using Chemagic Magnetic Separation Module I (MSM I) (Chemagen Biopolymer-Technologie AG, Baeswieler, Germany) according to the manufacturer's instructions. DNA from buccal swabs (Eurotubo Cytobrush, sterile, 200mm, Danlab, Helsinki, Finland) was extracted using QIAamp DNA Mini Kit (Qiagen).

Retinal samples from an Australian Cattle Dog (Study I and IV) and Swedish Vallhunds (Studies III and IV) were transferred to RNAlater stabilization and storage solution (Life Technologies). RNA was extracted using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions.

DNA and RNA concentrations were measured using Nanodrop ND-1000 UV/Vis Spectrophotometer (Nanodrop technologies, Wilmington, Delaware, USA) and the samples were stored at -20°C (DNA) or -80°C (RNA).

### **3.3 Clinical and laboratory studies**

Phenotyping was based on detailed clinical studies (II and III) in Dandie Dinmont Terrier and Swedish Vallhunds, as no previous published clinical

data was available. Other phenotypes (I and V) in the Papillon, Phalène and Norwegian Elkhound were based on ophthalmological reports from ECVO panelists. The clinical studies were performed using several methods including electroretinography (ERG), fundus photographs, gonioscopy, histopathologic examination, immunohistochemical staining, indirect ophthalmoscopy, laser scanning confocal microscopy light and fluorescent microscopy, neuro-ophthalmic examination, serum vitamin E measurements, slit lamp biomicroscopy, and tonometry. Different methods were used in each study.

**Papillon and Phalène (Study I):** Phenotyping was based on ophthalmological reports. All affected dogs were eye examined by ECVO panelists at least once. The affected dogs represented typical clinical signs of late-onset PRA.

**Dandie Dinmont Terrier (Study II):** A complete ophthalmic examination was performed on 18 Finnish Dandie Dinmont Terriers (3-13 years) without glaucoma, including neuro-ophthalmic examination, tonometry, indirect ophthalmoscopy, slit lamp biomicroscopy and gonioscopy. A detailed description the clinical examination is provided in Study II.

The anterior width of the ciliary cleft and the total distance from the origin of the pectinate ligament to the anterior corneal surface were subjectively evaluated. The ratio was used as an estimate of the RWOCC, which was graded as open, slightly narrow, narrow or closed.

The degree of the pectinate ligament dysplasia was categorized on the basis of the ECVO ([www.ecvo.org](http://www.ecvo.org)) guidelines in both eyes. The ICA was judged to be affected by PLD, when it exhibited abnormally broad and thickened pectinate ligament fibres or solid sheets of pectinate ligament tissue, with or without "flow holes" over 25% or more of the circumference. Dogs with PLD were graded 0-3 on the basis of the percentage of dysplasia present (Study II/Table 1). Intraocular pressure was measured on undilated eyes from all 18 unaffected dogs and post-dilation from 16 dogs.

The association between the PLD and ICA narrowing and age, possible PLD or the narrowing of ICA and their association to with gender and the association between PLD and glaucoma were evaluated. Detailed statistical analysis can be found in Study II.

**Swedish Vallhund (Study III):** All SVs were examined by indirect ophthalmoscope and with slit-lamp biomicroscopy. Fundus photographs were taken in selected dogs. All ocular abnormalities were documented. ERG was recorded in 15 SVs. Ocular histopathologic examination was performed in 8 SVs. The cell morphology and the localization of protein expression within the retina were studied. The immunohistochemical staining of retinal sections were performed with antibodies listed in Study

III/Table S1. Tissues were evaluated using light and fluorescent microscopy and imaged by laser scanning confocal microscopy. Serum vitamin-E measurements were performed in 19 dogs, including normal and affected SVs as well as mixed breed colony dogs. Detailed measurement techniques, histology and the imaging protocols are described in Study III.

To identify any possible environmental risk factors contributing to the development and progression of the disease, a short questionnaire was distributed to dog owners and breeders including inquiries about husbandry practices and previous occurrence of any systemic or ocular disease. Additionally, visual performance was assessed by distributing of a short questionnaire among 40 owners of affected SVs in Finland. Specifically, the questions concerned the owner's perception of the onset and severity of visual deficits observed in the dogs.

**Norwegian Elkhound (Study V):** Phenotyping was based on ophthalmological reports. All affected dogs were eye examined by ECVO panelists at least once. The affected dogs represented symptoms of primary glaucoma with significantly elevated IOP, with various degrees of optic nerve atrophy and cupping, and a progressive vision loss. Secondary clinical signs included lens luxation, corneal stromal edema, Haab's striae, keratitis, vitreal syneresis and retinal degeneration.

### 3.4 Genetic analyses

Several different statistical approaches and genetic methods were used in this study. Statistical approaches included genome wide association analysis (GWAS), linkage and combined association and linkage analysis. Genetic approaches included several molecular biology protocols such as SNP arrays, iPLEX SEQUENOM MassARRAY with custom SNPs, fragment analysis, exome and targeted resequencing, Sanger sequencing and RNA studies. Detailed descriptions of the methods and related statistical approaches can be found in each separate study.

**Genome wide association study (GWAS) (Studies I, II, IV, V):** The GWAS genotyping was performed using Illumina's CanineHD BeadChip with 173,000 markers (Study I and V) or Illumina Canine SNP20 BeadChip arrays with 22,000 markers (Studies II and IV) (San Diego, CA, USA). Genotyping was performed in our core facility at the FIMM Technology Center (Study I, II and IV) or at Geneseek (Lincoln, NE, USA, Study V).

**Fine-mapping (Study II):** Fine mapping was performed for 190 DDTs including 33 cases and 157 controls, using 110 additional custom SNP markers (~1 marker/100 kb) between 18 Mb to 29 Mb on CFA8 using iPLEX SEQUENOM MassARRAY platform (San Diego, CA, USA).

**Fragment analysis (Study III):** The candidate genes (*BEST1*, *CNGB3*, *NPHP4*, *PDE6B*, *PDE6A*, *PRCD* and *RPE65*) were studied using a fragment analysis and a pooling method with flanking microsatellite markers. The case (n=11) and control (n=11) DNAs were pooled and fragment sizes were compared between the case and control pools. The markers, where differences between the pools were found, were amplified and analyzed separately.

**Sequencing:** Three sequencing methods, exome sequencing, targeted resequencing and traditional Sanger sequencing were utilized in this study to identify the causative variants. This was followed by data analysis and filtering according to the suggested mode of inheritance. Sanger sequencing was used to genotype individual SNP, variants and coding regions of the candidate genes.

**Exome sequencing (Study I):** Exome sequencing was performed for a trio of Phalènes, including an affected proband and healthy parent dogs (Study I/ Fig. 1). The coding sequences were captured using Agilent's Canine Exome Capture Kit (Agilent, Santa Clara, CA, USA) and paired-end sequenced with the Illumina HiSeq2000 sequencing machine in our core facility at the FIMM Technology Center, according to manufacturer's instructions.

**Targeted resequencing (Study IV):** The associated region of 6.1 Mb on CFA17 was targeted and resequenced from four affected and four healthy control SVs. The regions were captured using custom designed probes for the build 2.0 of the canine genome reference sequence with the Roche Nimblegen solution based capture method. The capture was performed according the manufacturer's instructions and the paired-end sequencing was performed using the Illumina HiSeq2000 in our core facility at the FIMM Technology Center, according to manufacturer's instructions.

**mRNA studies:** The entire *CNGB1* mRNA was amplified in a retinal sample from an Australian Cattle Dog, free of PRA (Study I). The sequence was stored in the NCBI GenBank database (XM\_848817.1). The *MERTK* mRNA was sequenced from one unaffected Australian Cattle Dog and two retinopathy affected SVs (Study IV). RT-PCR was performed for candidate genes *MERTK*, *NPHP1*, *ANAPC1* and *KRCC1* (Study IV) in four affected SVs and two unaffected dogs, Australian Cattle Dog and Belgium Shepherd. *GAPDH* housekeeping gene was used as a normalization control. More detailed description about the RT-PCR can be found from study IV.



**Sanger sequencing (Studies I, II, IV and V):** Sequencing primers were designed using Primer3 software (biotoools.umassmed.edu/bioapps/primer3\_www.cgi). Reference sequences were obtained from the NCBI database (www.ncbi.nlm.nih.gov/). Detailed primers, PCR protocols, and sample and variant lists are described in each separate study. The Sanger sequencing in each separate study is described in the following chapters.

**Study I:** The *RPGRIP1* gene was studied as a candidate gene by sequencing the known insertion mutation<sup>172</sup>. The SNP on CFA1 (BICF2P839236) showing the strongest association was amplified in six PRA affected and 168 control Papillons and Phalènes. An indel in the *CNGB1* and a non-synonymous variant in *OGFOD1*, identified through the exome sequencing, were amplified from 6 PRA affected and 150 Papillons and Phalènes and one Rough Collie (*CNGB1*) and from 6 PRA affected and 14 control Papillons and Phalènes and one Rough Collie (*OGFOD1*). The *CNGB1* variant was further sequenced from 121 dogs from other breeds affected with PRA or retinal degeneration, representing 44 breeds and 334 healthy control dogs from 10 breeds (Study I/Table S3).

**Study II:** The glaucoma locus on CFA8 was tested in three other breeds affected with glaucoma or PLD by sequencing the three SNPs with the strongest association (Study II/Table S3). In addition to candidate loci the coding sequence and flanking splice sites of candidate genes *CTAGE5*, *FBXO33*, *LRFN5*, *PNN* and *TRAPPC6B* were sequenced.

**Study IV:** A replication study for the SNP (BICF2G630207991) on CFA17 was performed by genotyping it in additional 34 cases and 26 controls. In addition, the variants (n=30) identified on the basis of the targeted resequencing were genotyped in a large sample cohort of SVs (Study IV/Table 1) and from 8 unaffected dogs from 2 other breeds, Finnish Lapphund and Whippet, to study breed specificity.

**Study V:** The coding regions and splice sites of *ADAMTS10* were sequenced in four NE cases, four NE controls (unaffected at >8 years), and in one unaffected Rough Collie. The identified candidate mutation was validated in 596 NEs, including 7 additional cases. In addition, the mutation was studied in 71 dogs from 17 other breeds affected with POAG, PCAG or PLD and in 115 unaffected dogs from 6 breeds (Study V/Table S1).

### 3.5 Statistical analyses

In GWAS studies a case-control association test was performed using PLINK 1.07<sup>307</sup>. Quality control procedures were included when analyzing the data. Only SNPs that met the Hardy-Weinberg expectations of  $P \leq 0.0001$ , had  $\geq 95\%$  genotyping rate and a minor allele frequency (MAF) of 5% were included in the analysis. Significance values from these analyses were used to generate a whole-genome association plot using the statistical package R<sup>308</sup>. Identity-by-state (IBS) clustering and CMH meta-analysis (PLINK) were used to adjust for population stratification (Study II and V). Genome-wide corrected empirical p-values were determined by applying 100,000 (Study I and V) or 50,000 (Study II and IV) permutations to the data. In addition the data was analysed using a R-implemented GenABEL<sup>309</sup> and Gapit<sup>310,310</sup> analysis packages. The build 2.0 (Study I and II) or 3.1 (Study IV and V) of the canine genome reference sequence was used in the studies.

The fine-mapping data (Study I) was analyzed with PLINK 1.07<sup>307</sup> with at least 65% genotyping rate and MAF 5 %, resulting in the exclusion of 16 SNPs out of 110 and 20 individuals out of 190. Association and haplotype analysis for 3-SNP and 5-SNP haplotypes were performed using PLINK 1.07 sliding window option<sup>307</sup>.

A linkage analysis (Study I) was performed using the joint family-based linkage and association analysis program Pseudomarker<sup>311</sup>. The family-based analyses were performed under a recessive inheritance model, and included a parametric single-point linkage test, association analysis (LD|Linkage) and joint analysis (LD+Linkage).

Sanger sequence analysis was performed using Variant Reporter (Applied Biosystems, Foster City, California, USA) or Sequencher softwares (Gene Codes, Ann Arbor, MI, USA) and the fragment analysis data (Study III) was analyzed using a Peak Scanner™ Software v1.0 (Applied Biosystems).

The effect of the variants on the protein was predicted using bioinformatics tools Polyphen2.0<sup>312</sup> and SIFT<sup>313</sup>. The presence of non-synonymous variants in the exonic enhancer regions in Study V was evaluated using ESE-finder<sup>314</sup>.

The next generation sequencing data analysis for exome and targeted data included quality control, alignment to CanFam2.0 (Study I) or to CanFam3.1 (Study IV) reference sequences, variant calling with GATK<sup>315</sup> and Samtools<sup>316</sup> softwares and annotation of the variants to NCBI, Ensembl and UCSC databases with ANNOVAR<sup>317</sup> (Study I) or SnpEff<sup>318</sup> (Study IV) and an in-house R-script. Pindel program was used to identify structural variants<sup>319</sup>.

The data was then filtered assuming a recessive mode of inheritance using in-house R-scripts. In Study IV to identify the case-specific variants, the data was filtered against the controls and 16 other dogs from two

breeds, Dandie Dinmont Terrier and Staffordshire Bull Terrier. To study the CNVs and repeat elements (SINE, LINE), a heatmap analysis was performed by comparing normalized read depth in each position between cases and controls to study the possible lack or enrichment of the read depth. A more detailed description of the analysis pipeline can be found in Studies I and III.

To compare the vitamin-E levels, a Wilcoxon rank sum test was performed to compare the values between groups (Study III).

The statistical analysis of the clinical data in Study II and the qPCR data in study IV were performed using SPSS statistic package<sup>320</sup>. In study II Spearman correlation was used to calculate the association between PLD and ICA narrowing and age and PLD or the narrowing of ICA and association with gender was evaluated using non-parametric Kruskal-Wallis test. The association between PLD and glaucoma was determined using Chi-square test. In study IV the comparative  $\Delta\Delta\text{Ct}$ -method was used to determine relative expression differences. Statistical significance of the expression differences was calculated by using the Student's t-test on normalized mean cycle threshold (Ct) -values.

The relative risks were calculated using R-implemented epitools package<sup>321</sup> in Studies I, III-V, and a Fisher's exact test was used to calculate the association of the candidate variants in a larger sample cohort in Studies I, III, and V.

## 4 RESULTS

### 4.1 Mutation in the known human RP gene, *CNGB1*, in PRA affected Papillons and Phalenes (Study I)

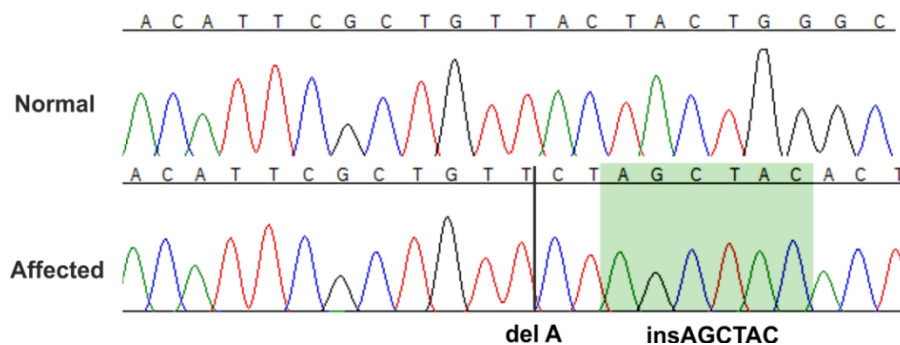
A high prevalence of PRA was noted among the breeds. A pedigree was drawn around the affected dogs to evaluate the inheritance model for study design (Study I/ Fig. 1). At the same time an affected dog was gene tested as a homozygous for the known *RPGRIP1* mutation reported by Mellersh et al.<sup>172</sup>. Because of this, five additional cases and 146 controls were screened for the *RPGRIP1* mutation, but although a high carrier frequency (17.1 %) was found in the breeds, the rest of the affected dogs did not carry the mutation, suggesting another genetic cause. This prompted us to perform a GWAS combined with an exome sequencing to map the PRA locus and, identify the mutation in our small study cohort with 6 cases and 14 controls.

The strongest association in GWAS was identified on CFA2 at 61.4 Mb ( $p_{\text{raw}}=4.7 \times 10^{-6}$ ,  $p_{\text{genome}}=0.1$ ). Another tentative region was found in CFA1 (70.3-77.6 Mb) ( $p_{\text{raw}}=7.0 \times 10^{-6}$ ,  $p_{\text{genome}}=0.2$ ). Re-analysing the data by adjusting for the strongest SNP on CFA2 ( $p=0.9$ ) or genotyping the SNP with the strongest association on CFA1 ( $p=6.97 \times 10^{-5}$ ) in a larger sample cohort did not improve the original association (Study I/ Fig. 2A). The association results were confirmed by a joint family-based linkage and association analysis using the Pseudomarker program<sup>311</sup>. The joint analysis identified the most highly linked region covering CFA2 61.1-64.9 Mb, ( $p_{\text{linkage}}=1.0 \times 10^{-6}$ ,  $p_{\text{association}}=8.7 \times 10^{-6}$  and  $p_{\text{joint analysis}}=1.9 \times 10^{-7}$ ) (Study I/ Fig. 2B). A homozygous haplotype (1.9 Mb) was shared by cases with recessively segregating markers between 61.4-64.5 Mb, including 37 genes (Study I/ Fig. 2C).

The exome sequencing data identified altogether 129,590 single nucleotide variants (SNVs) and 25,905 indels across the trio. More information about the exome sequencing results (read depth, on target coverage etc.) can be found in Study I/ Table S2. After filtering the data for the trio according to a recessive mode of inheritance, 204 SNVs and 9 indels remained. Two coding variants were located in the associated region on CFA2: a 1-bp deletion followed by a 6-bp insertion in the *cyclic nucleotide gated channel beta 1* (*CNGB1*) gene (c.2685delA2687\_2688insAGCTAC) (Study I/ Fig. 2D) (Fig. 10) and a non-synonymous mutation in the *2-oxoglutarate and iron-dependent oxygenase domain containing 1* (*OGFOD1*) gene (c.1128G>A). The indel variant in *CNGB1* causes a frameshift and a premature stop-codon p.Tyr889Serfs\*5 in an evolutionarily conserved region (Study I/ Fig. 3A, B),

while the missense mutation p.M376I in *OGFOD1* was predicted to be benign. No case-specific variants were found on CFA1.

Based on the position of the *CNGB1* indel and the formation of a frameshift, followed by a premature stop codon, it is hypothesized that the mRNA is subjected to nonsense mediated decay (NMD). NMD eliminates the production of mRNA that contains premature translation termination codon in a certain position before the next exon-exon junction and codes for nonfunctional polypeptide<sup>322,323</sup>. However, the NMD hypothesis was not tested due to a lack of tissue samples, but we were able to show that *CNGB1* is expressed in the normal canine retina.



**Figure 10.** An indel mutation in the *CNGB1* gene with a 1-bp deletion and a 6-bp insertion in the affected dogs.

To gather further evidence for the *CNGB1* mutation, six additional PRA affected dogs, four obligatory carriers from the pedigree (Study I/ Fig. 1) and 145 randomly selected Papillons and Phalènes were genotyped. All affected dogs were homozygous and carriers heterozygous for the mutation. Four additional homozygous dogs were found among the 145 dogs. Of these dogs, two were taken to the ECVO panelist and were also found to be clinically affected. However, the owners had not noticed any abnormalities in the dog's behavior, indicating that a dog manages well even with impaired vision, as previously described<sup>145</sup>. Two other homozygous dogs had been ophthalmoscopically examined at a very young age (1-2 years), and it is likely that the clinical signs are not present at this age. Based on the genotyping of a larger sample set a 17.2 % carrier frequency (25/145) was indicated ( $p_{\text{Fisher}}=1.4 \times 10^{-8}$ ). The mutation was screened for additional 334 healthy dogs from 10 other breeds without PRA and in 121 dogs from 44 other breeds affected with PRA or retinal degeneration, but the indel was not present in any of them, indicating that the *CNGB1* mutation is causal and breed-specific in Papillons and Phalènes (Study I/ Table S3).

The *CNGB1* gene encodes a rod photoreceptor cyclic guanosine monophosphate-gated (cGMP) channel  $\beta$ -subunit, which is part of the cyclic nucleotide gated (CNG) channel<sup>324-326</sup>. The phototransduction

cascade is mediated by the CNG cation channel, which is directly gated by cGMP<sup>327</sup>. The gene is expressed in multiple different tissues, including rod cells in the retina<sup>328,329</sup>, making it a highly relevant gene for the studied phenotype.

## **4.2 Primary closed angle glaucoma in Dandie Dinmont Terriers maps to a syntenic region of the human chromosome 14 with several glaucoma loci (Study II)**

The Dandie Dinmont Terrier (DDT) is a hunting terrier with a very long body, short legs, and a distinctive "top-knot" of hair on the head. It was named after a character in a novel written by Sir Walter Scott in 1814. All the Dandies around today descend from a dog found in a trap on the Duke of Buccleuch's estate - in 1839. Today DDTs are rarely used as a working terrier, but still make good companion dogs (Fig. 11). Breed clubs in the USA, UK and Finland contacted us about the increasing prevalence of the disease in the breed, suspecting a genetic contribution. In collaboration with the breed clubs, a genetic study was embarked on and DNA samples were collected from affected and unaffected DDTs around the world.

The clinical results revealed that the average age at the glaucoma diagnosis was 7.7 years and that several ocular abnormalities concerning ICA and PL structures were observed in the majority of the studied unaffected dogs. PLD was diagnosed in 72.3 % of the dogs and abnormal ICA width was found in 50 % of the dogs (Study II/Table 2). In the dogs with normal ICAs, PL was mostly normal with some slight changes in less than 50% of the circumference of the PL (<50%). Four of the studied dogs had no ocular changes with both PLD and ICA found to be grade 0 (Study II/Table 2). Fundi were normal in 14 of the studied dogs. Four dogs had a slight arteriole narrowing on the periphery of the retina, which is probably due to the age of the dogs (8-13 years). No association between age or gender and PLD or ICA narrowing was observed ( $p_{\text{PLD}}=0.45$ ,  $p_{\text{ICA}}=0.71$ ,  $p_{\text{PLD}}=0.40$ ,  $p_{\text{ICA}}=0.77$ , respectively). PLD was suggested as a risk factor for glaucoma development ( $P=0.05$ ).



**Figure 11.** The Dandie Dinmont Terrier is a terrier with very long body, short legs, and a distinctive "top-knot" of hair on the head.

The GWAS identified a 9.5 Mb associated region on CFA8 ( $p_{\text{raw}}=5.8 \times 10^{-6}$ ,  $p_{\text{genome}}=5.5 \times 10^{-3}$ ), ranging from 20.04 Mb to 29.5 Mb and a further CMH meta-analysis performed to correct for the stratification confirmed the association ( $p_{\text{raw}}=2.21 \times 10^{-5}$ ,  $p_{\text{genome}}=0.02$ ) (Study II/Fig. 1A-C).

Fine-mapping was performed in a replication cohort and identified the best association at 22.7 Mb ( $p=6.3 \times 10^{-5}$ ). When the whole dataset was combined, the strongest association was found for the SNP, BICF2P1308530 T>C (22.7 Mb). Based on the allele frequencies, the T allele was found in 52 % of the cases compared to 14 % in controls ( $p=5.3 \times 10^{-9}$ , OR=6.6). Homozygosity for the T allele increased the risk 32-fold (95% C.I. 3.7-280.8, 19% frequency in cases and 0.7% controls). The fact that the risk increased with homozygosity suggests a recessive inheritance possibly with a reduced penetrance as not all offspring born to affected parents are affected.

A haplotype analysis with a 5-SNP window narrowed the locus to 1.6 Mb between 22.0 and 23.6 Mb ( $p=1.6 \times 10^{-10}$ ) (Study II/Fig. 1D). Besides this region, three other significant associations at 19.7 Mb ( $p=2.8 \times 10^{-6}$ ), 25.57 Mb ( $p=6.6 \times 10^{-5}$ ) and 26.9 Mb ( $p=1.0 \times 10^{-6}$ ) were found (Study II/Fig. 1D).

The 1.6 Mb locus contained 7 genes of which *CTAGE5*, *FBXO33*, *LRFN5*, *PNN*, and *TRAPPC6B* were selected for candidate gene screening. Sequencing revealed six coding and 12 intronic variants (Study II/Table S4). However, none of the variants was case-specific and are therefore unlikely to be causative for the disease. The non-causative role was further supported by the bioinformatics prediction tools (Study II/Table S4). The three most highly associated SNPs were genotyped in four other breeds affected with glaucoma or PLD, but no association with either phenotype was observed in any of the breeds (Study II/Table S3).

The CFA8 locus is syntenic to human chromosome 14, which has been associated with POAG and PCG, but not PACG (Fig. 12).

### 4.3 A unique form of retinopathy in the Swedish Vallhund is caused by upregulation of *MERTK* (Studies III and IV)

Stage 1 was characterized by a diffuse multifocal red or brown discoloration of the tapetal fundus, but no visible lesions in the non-tapetal fundus or clinical signs of vision loss (Fig. 13 D) were observed compared with a normal fundus (Fig. 13 C). A total of 34 dogs were seen with a mean age of  $4.3 \pm 3.7$  years, but these initial signs were noted as early as 1.9 months and as late as 17.8 years of age (Study III/ Fig. 2D-F).

Stage 3 was characterized by more diffuse retinal thinning affecting most of the tapetal fundus (Fig. 13F). The dogs suffered from loss of night-vision and severely impaired day-vision. Some dogs were assessed as



completely blind by their owners. These changes were diagnosed in seven dogs with a mean age of  $12.2 \pm 2.6$  years, but were also observed as early as 9.2 years and as late as 15.4 years (Study III/Fig. 2J-L, Fig. 4B).

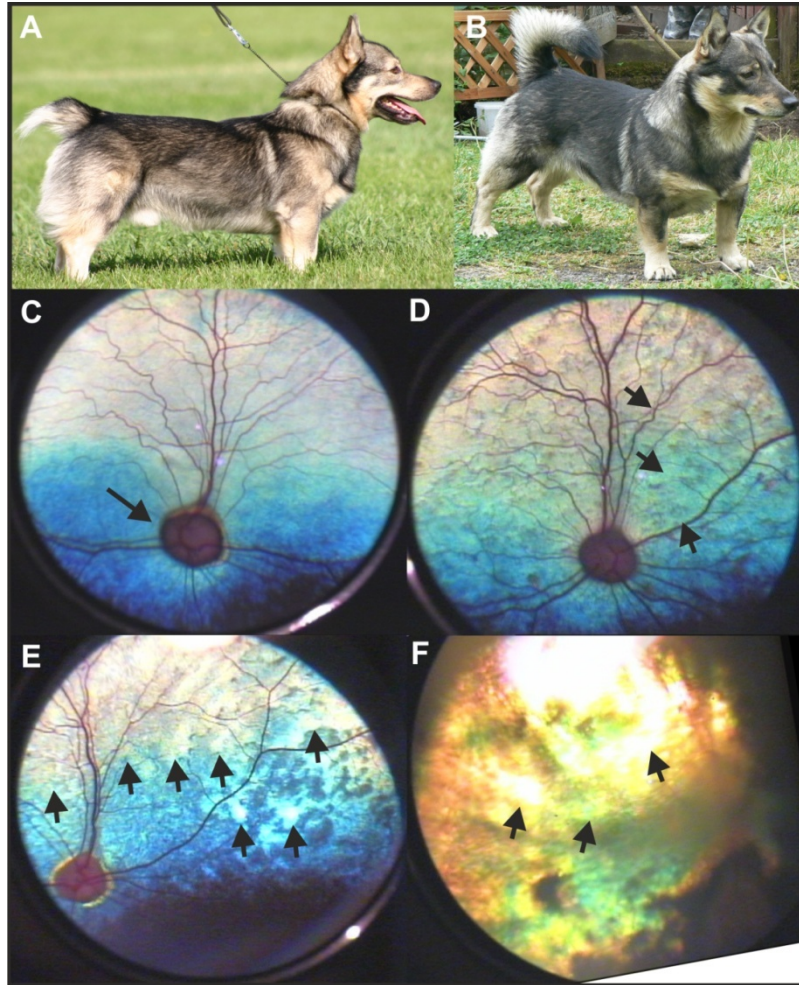
A total of five dogs were followed at stage 1 for a minimum of 0.9-2.3 years without any signs of progression to subsequent disease stages (Study III/Table 2). The precise duration could not be estimated as the pet population was distributed across several continents. One notable example is a dog that never progressed beyond stage 1 although was repeatedly examined until the age of 17.8 years. A total of four dogs were followed at stage 2 for a period of 1.8-3.9 years without any signs of progression to subsequent disease stages (Study III/Table 2). A total of ten dogs showed progression to subsequent disease stages (Study III/Table 1).

ERG revealed a decrease in both rod and cone photoreceptor-mediated functions at stages 2 and 3 (Study III/Fig. 3). The owners' observations that night-blindness precedes loss of day-vision was supported by the ERG indicating that cone function is lost more slowly than the rod function.

An accumulation of autofluorescent material in the subretinal space and within the RPE was noted (Study III/Fig. 4E, F). The presence of this material was more excessive than is seen with other forms of PRA or in normal aging. The acid-fast positive, ceroid/lipofuscin-like material was likely to be the source of the red or brown discoloration seen in the tapetal fundus at stage 1.

Other retinal alterations were found in 14 of the studied dogs including retinal scars and fold and 'bull's eye' lesions consistent with multifocal acquired chorioretinopathy (MAC). Detailed description of other unrelated findings can be found in Study III.

The pedigree suggested a strong genetic contribution (Study III/Fig. 5). An autosomal recessive mode of inheritance was supported by the lack of sex predisposition, and the observation that affected dogs can originate from the breeding of non-affected dogs. However, there are litters with affected parents but not all offspring develop the disease. This may indicate a reduced penetrance or that the offspring have not been ophthalmoscopically examined and may in fact be affected. It is also possible that the offspring are too young to have developed the clinical signs.



**Figure 13.** The Swedish Vallhund is a small spitz-type herding breed with different coat colors and a **A)** bob or **B)** long tail. **C)** Normal appearance of the ocular fundus with well-developed retinal vasculature, and the optic nerve head (arrow) **D)** Stage 1 is characterized by multifocal red/brown discoloration of the tapetal fundus **E)** In Stage 2 signs of degeneration, indicated by multifocal, geographic thinning, are present **F)** In Stage 3 more diffuse retinal thinning is observed as large, bright (hyperreflective) regions affecting most of the tapetal fundus.

A panel of known canine retinal degeneration genes *bestrophin* (*BEST1*)<sup>197-199</sup>, *cyclic nucleotide gated channel beta 3* (*CNGB3*)<sup>207</sup>, *nephronophthisis 4* (*NPHP4*)<sup>166,167</sup>, *phosphodiesterase 6B, cGMP-specific, rod, beta* (*PDE6B*)<sup>72</sup>, *phosphodiesterase 6A, cGMP-specific, rod, alpha* (*PDE6A*)<sup>80</sup>, and *retinal pigment epithelium 65* (*RPE65*)<sup>330</sup> were excluded as causative (Study III/Table S3), except for the *progressive rod-cone degeneration* (*PRCD*)<sup>63</sup> because of marker monomorphism, and a genome wide approach was initiated to map the disease locus.

Based on the GWAS, a tentative locus on CFA17 ( $p_{\text{raw}}=7.7 \times 10^{-5}$ ,  $p_{\text{genome}}=0.13$ ) was identified (Study IV/Fig. 1A). Genotyping of the SNP with the strongest association in a larger replication cohort supported the association ( $p_{\text{repl}}=2.7 \times 10^{-5}$ ) (Study IV/Fig. 1A). When analyzing the GWAS and replication cohort together, an association significant at a genome-

wide level was identified at CFA17 ( $p_{\text{comb}} = 4.3 \times 10^{-8}$ ) with increased risk for homozygosity (OR=11.2) (Study IV/Fig. 1A).

The associated region includes a 6.1 Mb homozygous risk haplotype in the cases spanning from 37.5 Mb to 43.60 Mb (Study IV/Fig. 1B). The region contains 102 genes, of which *anaphase promoting complex subunit 1* (*ANAPC1*)<sup>331</sup>, *c-mer proto-oncogene tyrosine kinase* (*MERTK*)<sup>332-341</sup> and *nephronophthisis 1* (*NPHP1*)<sup>342,343</sup> have previously been associated with retinal disease or retinal function (Study IV/Fig. 1C). Sequencing the coding regions and splice sites revealed three non-synonymous coding variants, p.A369P in the *MERTK*, p.H86R in the *NPHP1* and p.V734I in the *ANAPC1* (Study IV/Table 1).

The *MERTK* p.A369P and the *ANAPC1* p.V734I variants were predicted to be benign while the *NPHP1* variant was predicted to be pathogenic. The multiple alignments did not support the *MERTK* or the *ANAPC1* pathogenicity either. Furthermore, they did not fully segregate with the disease in SVs and were present in other unaffected breeds, suggesting that they were not causative.

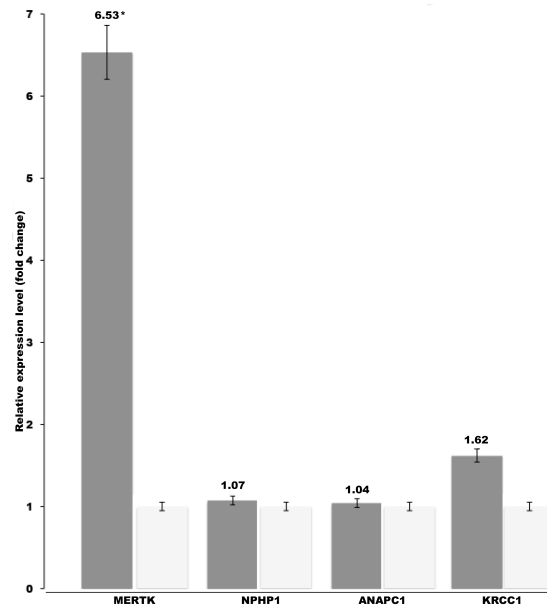
The targeted resequencing identified altogether 408 SNVs and 70 indels shared between the SV cases (Study IV/Table S1). Only one additional new case specific coding variant, p.L97R, in the *lysine-rich coiled-coil 1* (*KRCC1*) gene was found. However, further analysis of this variant in additional SVs and in other unaffected breeds did not support its segregation with the disease (Study IV/Table 1).

Among the non-coding variants, 30 were selected across the region. The variants were categorized on the basis of their type and position. Non-synonymous variants were prioritized first, followed by variants in the non-coding RNAs, UTR regions, and conserved intergenic and intronic regions, respectively. No segregation was identified in any of them (Study IV/Table 2). Possible variations in the CNV and the other repeat regions, SINE and LINE, were studied on the basis of a heatmap analysis, but no case specific differences were noted in the read depth.

Based on our analysis of the GWAS and targeted sequencing data the SNP, BICF2G630207991, positioned in the intron of *MERTK* was the most strongly associated marker. This marker was further tested in 400 SVs revealing a significant risk for homozygosity ( $p = 6.3 \times 10^{-27}$ , OR=18.5 with 95%CI=10.3-33.2) and a carrier frequency of 59.9 %.

Real-time PCR of *MERTK*, *NPHP1*, *ANAPC1* and *KRCC1* revealed a 6.5-fold upregulation of the *MERTK* in the affected dogs while no difference was found in the other genes (Fig. 14). This result indicates that a regulatory mutation outside the coding region results in overexpression of *MERTK*, which leads to retinopathy in the affected dogs.

*MERTK* was a highly relevant candidate gene, belonging to the TAM receptor tyrosine kinase family, and it has been shown to be expressed in the RPE plasma membrane<sup>344</sup> and be involved in apoptosis and inflammation<sup>345</sup>.



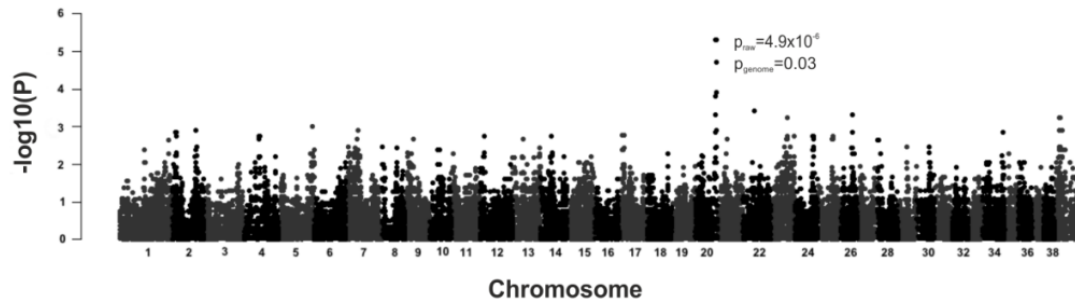
**Figure 14.** A specific overexpression of *MERTK* was found in the affected SVs when comparing the candidate *MERTK*, *NPHP1*, *ANAPC1* and *KRCC1* genes. The relative mRNA expression levels are represented as a fold change. Error bars denote the standard error of normalized Ct-values. \* $p \leq 0.0001$  (two-tailed t-test p-value).

#### 4.4 A novel missense mutation in human Weill-Marchesani syndrome gene, *ADAMTS10*, causes primary glaucoma in Norwegian Elkhounds (Study V)

The glaucoma phenotype in NEs was described long ago<sup>254,255</sup>, but genetic research has only now become possible with the advent of canine genomic tools. To map the disease locus, samples were collected in collaboration with ECVO panelists in Finland and the US from 16 affected and 596 control dogs. The clinical data of the affected dogs indicated bilateral primary glaucoma with significantly elevated IOP, between 25-86 mmHg (normal 10-20 mmHg) on average, at the age of 6.5 years. In addition, various degrees of optic nerve atrophy and cupping with a progressive vision loss were seen. Secondary clinical signs included lens luxation, corneal stromal edema, Haab's striae, keratitis, vitreal syneresis and retinal degeneration.

The GWAS revealed a 750 kb locus on CFA20 between at 53.0-53.8 Mb ( $p_{\text{raw}}=4.9 \times 10^{-6}$ ,  $p_{\text{genome}}=0.03$ ) (Study V/ Fig. 2A-C, S2) (Fig. 15). The locus is a known canine glaucoma locus, harbouring the known canine glaucoma gene *ADAMTS10*<sup>227</sup> (Study V/ Fig. 2D), which was selected for mutation screening. The gene's importance was further supported by its

association with glaucoma being consistent with the previously reported POAG phenotype of primary glaucoma in NEs<sup>254,255</sup>.



**Figure 15.** The GWAS mapped the primary glaucoma locus in NEs on CFA20.

Sequencing the coding regions and splice sites in four affected and five control dogs identified altogether ten variants; a non-synonymous variant c.1441G>A, p.A387T (Study V/Fig. 3A,B), five synonymous and four non-coding variants (Study V/Table S3). Only one synonymous variant, p.P171P, co-segregated with the non-synonymous variant. However, ESE-finder did not indicate its position to be in the exonic enhancer region. The non-synonymous variant was predicted to be pathogenic, which was further supported high evolutionary conservation between 75 species (Study V/Fig. S1).

*ADAMTS10* is a secreted glycoprotein<sup>346</sup> and belongs to a family of metalloproteinases that contribute to the dynamics of the extracellular matrix (ECM) composition, microfibril and collagen function and remodeling of the membranes<sup>247,346-350</sup>. It interacts with fibrillin-1 and localized to fibrillin-1 microfibril bundles<sup>247</sup>.

To gather further evidence for the p.A387T segregation with the disease phenotype, seven additional NE primary glaucoma cases, four obligatory carriers and 572 randomly selected unaffected NEs were genotyped. All but two of the cases were homozygous for the mutation. Unfortunately, we had a limited access to the health records and history of these two affected dogs and they may have had a secondary glaucoma with the primary underlying cause being missed at the end stage of the disease. All four obligatory carriers were heterozygous and based on the 572 NEs a 25.3 % carrier frequency (151/596) was indicated. Only one additional homozygous dog was found, which is now 5 years old without any signs of glaucoma, but the follow-up needs to be continued. These results support the segregation of the mutation in clinically confirmed primary glaucoma cases with a highly significant association between the mutation and the disease ( $P_{\text{Fisher}} = 3.5 \times 10^{-27}$ ). The breed-specificity of the p.A387T mutation was indicated by its absence in 71 glaucoma or PLD

## *Results*

affected dogs from 17 breeds and in 115 unaffected dogs from six other breeds (Study V/Table S1).

## 5 DISCUSSION

This study focused on four different eye disorders in five different dog breeds and made breakthroughs in describing the clinical phenotypes and identifying genes and loci for the diseases. It has succeeded in establishing remarkable resources for the characterization of canine vision disorders and in utilizing the recent genomic tools to map several new disease genes. The study demonstrates the feasibility of mapping genes in small populations, while also highlighting the challenges to the identification of causative mutations in extensive LD regions in small, inbred populations.

We have clinically described a unique type of retinopathy in the Swedish Vallhund and primary closed angle glaucoma in the Dandie Dinmont Terrier, neither of which has previously been described in the veterinary literature. Furthermore, we mapped three novel genes and a novel locus for the studied phenotypes.

This study is methodologically diverse and utilized various state-of-the-art approaches to identify the clinical and genetic causes. The study relies completely on samples collected from privately owned pet dogs worldwide. This required remarkable efforts in collaboration with breeders, breed clubs, and dog owners. Overall, thousands of samples from affected dogs across various breeds and eye conditions were collected in the DNA bank beyond the four phenotypes that were under the specific focus of this study.

An important aspect of this study involves intimate collaborations between the basic and clinical research including geneticists and the clinicians, both human and veterinary ophthalmologists. This network of collaboration enabled us to combine specific expertise in phenotyping and sample collection in multiple breeds and conditions utilizing privately owned pet populations. This collaboration extended beyond academics to dog owners and breeders worldwide who actively participated in the study and contributed to the establishment of relevant study cohorts. As a result we were able to describe clinically two unreported diseases in SVs and DDTs and map or identify the genetic causes.

Gene mapping relied on the state-of-the-art approaches in the field. We used different generations of SNP arrays and the next generation sequencing approaches were piloted with the establishment of proper bioinformatics analysis pipelines to discover mutations. Although the canine reference genome has improved significantly over the years it is currently based on the transcript sequences of a limited collection of selected tissues not necessarily relevant for this study (for example retinal transcripts have not been included). This proved to be one of the major

obstacles in this study and prevented comprehensive listing of all the possible variants in the target regions for further confirmation.

This study has several theoretical and practical implications. An important observation emerging from these studies identifies the genetic similarity of eye disorders across species. Three of the implicated canine genes have previously been associated with corresponding phenotypes in humans and rodents. In addition, the novel glaucoma locus in DDTs maps to a human chromosomal region that has been associated with glaucoma in numerous studies and patient cohorts. Moreover, canine genetics appear less complex with stronger founder effect with breed-specific traits compared with human studies. All affected dogs carry the same mutation and do not show significant locus heterogeneity. In contrast, many more mutations have been described in the corresponding human phenotypes, e.g. altogether six mutations have been found in the human *CNGB1*<sup>84,351-354</sup> and 12 mutations in the human *MERTK* patients<sup>332,334-341,355-358</sup>. Although we did not find locus heterogeneity in our phenotype, there are other PRA breeds with multiple causative loci. For example, PRA in the Golden Retriever, consists of at least three genetically different types<sup>63,62,118</sup>.

Although we were able to define the clinical phenotype and segregation pattern of the mutations in most of the affected dogs, some discrepancies remain. Some of these can be explained by the age of the dogs, as young dogs with the risk genotype may still develop the disease at older age. For example, four homozygous dogs were found among Papillons and Phalènes without reported signs of retinal degeneration. However, additional eye examinations revealed retinal degeneration in two of them, while the other two are still quite young for diagnosis. In the NEs, two cases without the homozygous mutation were found, suggesting a possible secondary disease or other genetic factors. Furthermore, it is possible that the observed reduced penetrance may be due to environmental factors or epigenetics, especially in SVs and DDTs, as not all the affected dogs were homozygous for the risk variant or had the phenotype despite having the risk genotype. The clinical follow up of these dogs would be of great importance.

We also observed some breed-specific differences in the phenotype. The affected NEs lack severe non-ocular signs that are commonly present in the WMS patients. This may be due to a lack of more detailed clinical examinations, which need to be carried out in future studies. The genetic test allows an early identification of genetically affected dogs that could be followed for years for a comprehensive clinical study.



## 5.1 Clinical and genetic characterization of two canine retinal degenerations reveal similarities with human retinal disease

This study describes the clinical and genetic characterization of two retinal diseases in Papillon and Phalène and Swedish Vallhund breeds. The two genes implicated for retinal degenerations, *cyclic nucleotide gated channel beta 1* (*CNGB1*) in the Papillons and Phalènes and *MER proto-oncogene, tyrosine kinase* (*MERTK*) in the Swedish Vallhunds are important for normal photoreceptor function, especially in rod and RPE cells.

In human patients with *CNGB1* mutations, several retinal abnormalities are reported, some at school age, but more commonly at around 30 years of age<sup>84,351-354</sup>. In a *Cngb1*<sup>-/-</sup> mouse model, the gene is important for the formation of the rod outer segment CNG channels, and a retinal degeneration is diagnosed<sup>359</sup>. However, the disease in mice progresses slowly and 80-90% loss of the rod responses is not detected until the age of 1 year<sup>360</sup>. PRA in Papillons and Phalènes is diagnosed at the middle age dogs and progresses similarly to humans and mice, further supporting the causative role of the *CNGB1*.

*CNGB1*-dogs provide a new large animal model for potential therapeutic purposes. A gene therapy has already been developed for the *Cngb1*<sup>-/-</sup> mice using AAV-vectors<sup>361</sup>. The gene therapy resulted in high level expression of the *CNGB1* protein leading to a delay in retinal degeneration and restored rod-driven light responses<sup>361</sup>. As the AAV-vector has been shown to work in canine models, a therapy for the *CNGB1*-dogs could be developed in a similar manner. A gene therapy has already been developed for the canine *CNGB3* gene in a canine model for achromatopsia<sup>303</sup> and as *CNGB1* and *CNGB3* are part of the CNG channels, the same therapy method may be useful in treating *CNGB1*-dogs.

In SVs, we described clinically a unique, breed-specific form of retinal disease with multifocal degeneration of the retina. Other reported retinal degenerations typically involve diffuse degeneration of the retina. Other specific features in the SV retinopathy include considerable variation in the age of onset and rate of progression not known to be present in any other breed. Furthermore, another unique detail was the abnormal accumulation of autofluorescence material in the RPE. Clinical observation and histopathological evaluation confirmed that the disease affects both photoreceptors and the RPE. However, the primary defective cell population cannot be determined with the limited material available. The discrepancies noted with respect to the rate of disease progression among Swedish Vallhund dogs suggest that genetic or environmental disease modifiers are likely to play a role in the pathogenesis. Retinal function is modified by environmental factors<sup>60,362-364</sup> and we investigated the role of

vitamin-E deficiency, but the results were inconclusive to in regard to the correlation with the clinical variability.

The SV retinopathy appears clinically and genetically unique. While other retinal degenerations are often shared at least among related breeds<sup>60,362-364</sup>, the SV retinopathy has not been described in any other breed and the *MERTK* locus was also mapped for the first time for canine retinal degeneration. It is possible that this relatively old breed originating from the Viking era between 8<sup>th</sup> to late 11<sup>th</sup> century has avoided mixing with other breeds, although Welsh Corgis may be created from SVs, according to the Swedish Vallhund breed club.

Although the actual causative mutation remains to be found, *MERTK* stood out as the most likely candidate gene based on its function and clinical significance in the retinal degeneration in other species<sup>333,337,339,355</sup>. Furthermore, we were able to show that the gene is upregulated in the affected SVs, indicating the presence of regulatory mutation. The upregulation was *MERTK* specific as no changes in expression levels of other candidate gene was found.

In human, mutations in *MERTK* have been associated with several recessive phenotypes<sup>332,334-336,340,341,355-358,365-369</sup>. Moreover, it has been shown that human patients show deposit of autofluorescence material, which may represent photoreceptor outer segment membranes, including lipofuscin, over-accumulating in the outer retina<sup>339,357</sup>.

Besides human, *MERTK* has been extensively studied in the Royal College of Surgeon (RCS) rats<sup>333,370</sup>. The rats present a progressive loss of photoreceptor cells due to abnormal phagocytosis of photoreceptor outer segments that normally should occur by the RPE<sup>333</sup>. This leads to the accumulation of photoreceptor outer segment (POS) debris between photoreceptors and the RPE<sup>370-373</sup>. Human and rat models have been associated with recessive loss-of-function mutations while our canine model represent a gain-of-function model with overexpression of the *MERTK* gene.

Although, we were not able to pinpoint the actual causative mutation, it is probable that the causative mutation is in LD with the identified marker, and the same carrier frequency might be seen also if the actual mutation was tested. However, the affected dogs present a novel model for the role of *MERTK* in the retinal biology. Whole genome sequencing might be considered in order to reveal the causative mutation. WGS has an advantage over the capture protocols as it does not exclude repetitive regions, which could be causal.

## 5.2 Canine glaucoma studies reveal a known human gene and a new candidate locus

This study describes the clinical and genetic characterization of primary closed and open angle glaucomas in the Dandie Dinmont Terrier and Norwegian Elkhound breeds, respectively. The *ADAMTS10* mapped in NEs has previously been associated with the canine POAG and is important in the glaucoma pathogenesis. Furthermore the CFA8 locus in DDTs is syntenic to human chromosome 14 containing multiple glaucoma loci. These genes and loci have been implicated in similar phenotypes in humans and rodents.

In DDTs, the clinical study revealed that the phenotype mainly resembles PACG with an onset at the middle age. This was the first study to describe the DDT phenotype in detail. However, the actual onset is likely to be earlier as most of the affected dogs visit a veterinary clinic at a later stage of the disease and initial clinical signs may have gone unnoticed. Congenital disease cannot be formally ruled out.

In DDTs a novel locus was mapped on CFA8 and was found to support a recessive inheritance mode, possibly with reduced penetrance. However, no causative variant was identified in any of the candidate genes. This locus is the second mapped canine glaucoma locus in addition to the Beagle locus on CFA20 with the *ADAMTS10* mutation<sup>227</sup>. More recently, two loci have been suggested in the Basset Hound on CFA14 and CFA24 near the genes *COL1A2*, *PEG10* and *RAB22A*, respectively<sup>229</sup>. However, the association signals were only tentative, suggesting too small a sample size, or too complex a phenotype, to reach the genome wide significance level, and no functional variant was found in the studied genes. In contrast, the CFA8 locus is strongly supported by the genetic data in our study.

Another glaucoma study was published in the Shih Tzu and Shiba Inu, in which three candidate genes were studied by genotyping SNP markers<sup>228</sup>. A significant association was found for the *SRBD1* gene, with two significant markers, a synonymous and an intronic SNP, which may serve as markers to evaluate the development of glaucoma in these two breed. *SRBD1* is located on CFA10, but outside our association signal, excluding the role of *SRBD1* in the DDT glaucoma.

The mapped DDT PACG locus shares synteny with a region on human chromosome 14. Chromosome 14 has been associated several times in human PCGs and POAG<sup>283,374-378</sup>, but not PACG. None of the human PCG or POAG loci on chr14 directly overlap with the canine locus mapped in DDTs. However, our data suggest a novel locus within the same glaucoma cluster for further studies in human cohorts.

In Norwegian Elkhounds a mutation in the *ADAMTS10* was found causative in primary glaucoma. The GWAS mapped the locus on CFA20 harbouring the *ADAMTS10* gene. The missense mutation in the NEs is

located in the metalloprotease domain and this may indicate that the pathogenesis of the primary glaucoma in NEs is different from the Beagle, as the p.G661R mutation is situated in the cysteine rich domain and is hypothesized to disrupt protein folding leading to instability<sup>227</sup>. The NE mutation changes a highly conserved residue in the metalloprotease domain, which plays a role in the remodeling of the connective tissue<sup>379</sup>.

In human, mutations in the metalloprotease domain lead to abnormalities in the cellular cytoskeleton. This may be due to abnormal interactions with the ECM<sup>379</sup>. Lens ligaments are comprised primarily of fibrillin-1 and the lens is maintained in its position by lens ligaments<sup>248</sup>. Fibrillin-1 is expressed in the outflow pathway of the aqueous humor and defect in it may lead to impaired aqueous humor flow<sup>249-251</sup>. The cytoskeleton abnormalities may result in defective microfibrils and glaucoma through fibrillin-1. Unfortunately, we did not have access to any tissue samples from the affected dogs to further investigate the functional consequences of the mutation for *ADAMTS10* and its pathway. Secondary lens luxation diagnosed in the affected NEs may be due to abnormal fibrillin-1 function as the *ADAMTS10* and fibrillin-1 interaction may be impaired causing disruption of the lens ligaments.

In humans, mutations in *ADAMTS10*<sup>379,380</sup>, *ADAMTS17*<sup>380</sup>, and in *fibrillin 1 (FBN1)*<sup>381</sup> have been associated with Weill-Marchesani syndrome (WMS) (OMIM 277600). WMS is a connective tissue disorder and characterized by several eye defects and other skeletal features<sup>382</sup>. Ectopia lentis is the most prevalent ocular defect though glaucoma is also diagnosed<sup>383</sup>.

Fibrillin-1 defects are thought to cause dysgenesis of the lens ligaments and ectopia lentis<sup>247</sup>. In dogs mutation in the *ADAMTS17* has been shown to cause ectopia lentis in multiple different breeds<sup>384</sup>. The ectopia lentis in dogs is probably caused by the fibrillin-1 defect as *ADAMTS17* and *ADAMTS10* are important for normal fibrillin function<sup>227,384,385</sup>. However, none of the affected NE dogs had any signs of primary lens luxation, although this should be expected based on importance of *ADAMTS10* and fibrillin connection. Access to tissue samples and histological analyses may give new insight into the possible lens zonular dysplasia in NEs.

Furthermore, therapy trials could be developed for NEs, although this might be problematic due to the late-onset of the disease. However, NEs provide a second large animal model, in addition to the Beagle, to study the *ADAMTS10* pathogenesis, its role in glaucoma and how fibrillins may influence the glaucoma development.

### **5.3 New tools and models for therapeutic trials, breeding programs and veterinary diagnostics**

This study has also several practical implications that have positive effects on breeding programs and veterinary diagnostics. First, two gene tests have already been developed for Papillons and Phalènes and NEs to help the breeders to avoid affected dogs. In both conditions, the carrier frequencies were high, 17 % and 25 %, respectively and the genetic tests can be utilized to eradicate the diseases in the breeds without compromising the breeds' genetic diversity by keeping the unaffected carrier dogs in the breeding populations.

Second, a marker based test can be developed for SVs while waiting for the discovery of the causative mutation. The prevalence of the SV retinopathy was almost 35 % and the carrier frequency for the best marker close to 60 %. For DDTs, the development of a marker test is more problematic. Although the risk for homozygous dogs was high (32-fold, with 95% C.I. 3.7-280.8), the breed is very small worldwide and largely affected with PLD in addition to glaucoma. Removing dogs from the breeding programs based on such high C.I. may be more harmful for the breed than allowing homozygous risk dogs. Importantly, it would be of great significance to identify the actual causative mutation to increase accuracy of the test and risk models in the DDT population.

High risk allele frequencies pose a challenge for the breeding programs as carrier-carrier matings cannot be avoided without compromising too much of the genetic diversity of the breeds. In SVs, it might be worth considering mixing the breed with other breeds to increase diversity and to reduce the frequency of the risk allele. This study demonstrates the harsh reality about the genetic structure of the breeds with extremely high-risk allele carrier frequencies, which may be impossible to eradicate from the breed without major changes in the breeding plans. New recessive diseases might become more frequent in the breed if too many carrier dogs are removed from breeding pools. It is also worth noting that there could be some differences in the accuracy of the marker and mutation tests, although canine LD is extensive.

Third, the genetic tests developed on the basis of this study can also be used as a part of the veterinary diagnostics to distinguish the genetically known diseases from each other and to test unknown conditions for the inclusion or exclusion of the gene. For example, PRA tests developed from this study could be utilized in differential diagnoses to distinguish genetically different PRAs commonly present in many breeds. This study has shown the benefits of the dog as a large animal model in genetic research. It has provided new tools for breeding and novel therapeutical models for medical research in humans. Furthermore building a collaboration network between genetics and clinicians has been a vital part of this study.

## 6 CONCLUSIONS AND FUTURE PROSPECTS

This study reveals significant new insights into the two of the most common hereditary ocular diseases causing blindness, retinal degeneration and glaucoma. The diseases are diagnosed in both humans and dogs and have an effect on the quality of life for humans while in dogs the diseases cause significant burden for the affected dogs and the dog owners. Irreversible blindness is the end point of the disease and no known cure exists at the moment for either disease. In addition, glaucoma is a painful disorder, if IOP is significantly elevated.

Furthermore, this study shows the benefits of the dog as an animal model. Instead of laboratory colonies, only privately owned pet dogs were used in this study. No affected dogs were bred for study purposes. The unique canine population structure facilitates genetic studies and substantial breakthroughs are possible in small sample cohorts. However, this necessitates efficient collaboration with breeders and private veterinarians. Based on the result two gene and one marker test have been developed. To best describe the significance of this study to the dog owners, a NE breeder wrote:

*“I would like to thank you and your team from the bottom of my heart for doing this research. Back in the 1980s I had a bitch who developed glaucoma at 2 years old. She was totally blind by 4 years old and although she lived a happy life to 13, it was absolutely devastating for all of us. Her grandsire on the dam's side was a Norwegian import, who himself had glaucoma so I now know that her sire must have also carried the gene. All we could do back then was avoid using that dog again. New breeders now don't remember those cases so the importance of finding out about glaucoma has become less to them. This research will mean that no dog and no owner should ever have to suffer like our dog did and we did. Once again thank you for all your hard work. I've lived with two dogs with glaucoma and never thought I would see this day. I am so thrilled.”*

This illustrates the importance of studying diseases among pet populations. The gene tests can now be used to help with the breeding and to avoid affected dogs. Furthermore, the carriers can be used in breeding programs to maintain the genetic diversity in the breed.

Collectively, these results give new insight into molecular pathways in retinal degeneration and glaucoma in dogs and show the power of dogs in genetic research of ophthalmological diseases. Our results implicate two retinal genes in the conditions and establish the affected breeds as potential models for gene therapy trials. The novel glaucoma locus in DTTs may also contain a yet unknown gene for human glaucoma,

advancing the understanding and treatment of the conditions in both species.

The identification of genes causative for canine vision disorders will also enable comparative studies across species, including humans, as all studied phenotypes are diagnosed in human patients. This study is likely to benefit the human medical research and possibly aid the development of new therapy methods.

While gene mapping is feasible in canine population, this study demonstrates also the challenge of extensive LD for the identification of a mutation in gene rich regions. Despite extensive efforts with state-of-the-art approaches, we could not find the mutation in the identified regions for two phenotypes. Therefore, two important major tasks remain in this study: the identification of the causative mutations for retinal degeneration in SVs and PACG in DDTs. Further studies into retinal degeneration of SVs should include the analysis of the retinal transcripts in affected and unaffected SVs. The aim is to obtain more detailed information on the retinal genes in a normal retina and to study the possible alterations between affected and unaffected SVs. As for DDTs, our existing targeted NGS data needs to be reanalyzed using the new canine reference sequence and annotation to identify the causative variant.

Other interesting studies would include further clinical studies in the NE glaucoma to investigate whether additional ocular or non-ocular abnormalities are present. For example, the lenses of the affected dogs should be followed to obtain information on possible signs of lens luxation. This would provide information on possible species-specific differences in the gene function and genotype-phenotype correlations of the mutations between dogs and humans.

Importantly, during this study a significant canine DNA bank was created holding thousands of samples from tens of different ocular phenotypes. This remarkable resource will benefit ongoing and future genetic studies in canine vision disorders to reveal new insights into the ocular and other diseases diagnosed in the man's best friend.

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